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INFLUENCES OF DOPAMINE AND NOREPINEPHRINE ON GONADOTROPIN RELEASE IN GOLDFISH, *CARASSIUS AURATUS*

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JOHN PHILIP CHANG

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **INFLUENCES OF DOPAMINE AND NOREPINEPHRINE ON GONADOTROPIN RELEASE IN GOLDFISH, *CARASSIUS AURATUS*** submitted by **JOHN PHILIP CHANG** in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

Abstract

The involvement of dopamine (DA) and norepinephrine (NE) in the regulation of gonadotropin (GtH) secretion in goldfish was studied by monitoring changes in serum GtH levels following pharmacological manipulations, and by studying the effects of DA and NE on GtH release by dispersed pituitary cells and/or pituitary fragments *in vitro*.

Results from experiments with intraperitoneal injections of drugs capable of blocking specific enzymes of catecholamine synthesis suggest that DA may inhibit GtH release in goldfish.

Intraperitoneal injections of DA or its agonist, apomorphine, decreased serum GtH levels in female goldfish. Intraperitoneal injections of the DA antagonists pimozide and metoclopramide increased serum GtH concentrations. Intraventricular (3rd cranial ventricle) injections of DA did not alter serum GtH concentrations. These results suggest that DA inhibits GtH release in goldfish by direct actions on gonadotrops.

Intraperitoneal injections of DA or apomorphine both reduced the elevated serum GtH levels caused by spontaneous release of GtH from a hetero-transplanted pars distalis. Preoptic lesions abolish an inhibitory hypothalamic influence on GtH release allowing prolonged spontaneous release of GtH; both DA and apomorphine caused a decrease in serum GtH levels in preoptic lesioned fish. Perifusion of dispersed goldfish pituitary cells or pituitary fragments with DA decreased the spontaneous release of GtH. These results indicate that DA inhibits spontaneous GtH release.

Intraperitoneal injections of des Gly^{10}, [D-Ala^{6}] luteinizing hormone-releasing hormone ethylamide (LHRH-A) caused an increase in serum GtH concentrations, but did not stimulate germinal vesicle migration or ovulation in gravid female goldfish. Intraperitoneal injections of pimozide or metoclopramide potentiated the action of LHRH-A to stimulate GtH release; the combined treatment of LHRH-A and pimozide or metoclopramide caused ovulation in gravid female goldfish. Intraperitoneal injections of DA, apomorphine, or bromocriptine (DA-agonist) blocked the LHRH-A induced elevation of serum GtH levels. *In vitro* perifusion of dispersed goldfish pituitary cells or pituitary fragments with DA abolished the LHRH-A induced increase in GtH release. These results indicate that DA directly inhibits the actions of GtH-releasing hormone and suggest that the preovulatory surge of GtH release is regulated by both stimulation by GtH-releasing hormone and

release from DA inhibition on GtH secretion. The DA inhibition on GtH release seems to be specific as intraperitoneal injections of phentolamine (alpha–antagonist), propranolol (beta–antagonist), or octopamine (sympathomimetic agent) did not alter normal serum GtH levels or the responses to LHRH–A.

In vitro perifusion with NE of dispersed pituitary cells obtained from female goldfish having regressed ovaries increased GtH release. Intraperitoneal injections of NE or clonidine (alpha–agonist), or intraventricular injections of NE increased serum GtH concentrations in female goldfish at times of year when the ovaries were regressed or at early stages of recrudescence, but had no influences at other stages of the ovarian cycle. in fish at early stages of ovarian recrudescence, the NE induced increase in serum GtH levels was blocked by simultaneous intraperitoneal injection of phentolamine. These results suggest that NE stimulates GtH release by alpha–adrenergic mechanisms at the pituitary and/or brain level in female goldfish in a sexually regressed or early ovarian recrudescence condition.

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List of Abbreviations

a) Abbreviations frequently used in text, tables and figures:

cAMP	adenosine cyclic monophosphate
DA	dopamine
DDC	diethyl-dithio-carbamate
GnRH	gonadotropin-releasing hormone
GRIF	gonadotropin release-inhibitory factor
GSI	gonadosomatic index
GtH	gonadotropin
h	hour
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LHRH-A	des Gly^{10}, [D-Ala^{6}] LHRH ethylamide
αMPT	alpha-methyl-para-tyrosine
NE	norepinephrine
6-OHDA	6-hydroxydopamine
PS	fish physiological saline
ug/g	ug/g body weight

b) Abbreviations occasionally used in tables and figures:

APO	apomorphine
bromo	bromocriptine
ip	intraperitoneal injection
iv	intraventricular injection
MET	metoclopramide
OCT	octopamine
PHEN	phentolamine
PIM	pimozide
PROP	propranolol
Hanks with BSA	Albumin (0.1% bovine serum albumin) supplemented Hepes-Hanks solution

I. General Introduction

Numerous studies on mammals have demonstrated that neurotransmitters influence luteinizing hormone (LH) release (for review see Krulich, 1979; Sawyer, 1979). Among the various neurotransmitters, the role of catecholamines in the neuroendocrine regulation of LH secretion has received the most attention and has been extensively reviewed (Gallo, 1980; Porter *et al.*, 1980; Barraclough and Wise, 1982). Results of experiments on the influences of catecholamines on LH release in mammals are briefly described below.

Depletion of norepinephrine (NE) or administration of alpha-adrenergic blockers inhibited pulsatile LH release in gonadectomized rats (Drouva and Gallo, 1977; Gnodde and Schuiling, 1976; Weick, 1978) and monkeys (Bhattacharya *et al.*, 1972), and the injection of the alpha-agonist clonidine reversed the cessation of pulsatile LH secretion in rats due to aging (Estes and Simpkins, 1982) or depletion of NE (Estes *et al.*, 1982). These results suggest that norepineprhine stimulates episodic LH release via alpha-receptors.

Norepinephrine also plays a stimulatory role in mediating the proestrous LH surge by stimulating alpha-receptors. Increased turnover of NE in the hypothalamus was associated with proestrous LH and luteinizing hormone-releasing hormone (LHRH) increases in rats (Rance *et al.*, 1981b). Furthermore, lesions in the ventral norepinephrinergic pathway (Martinovic and McCann, 1977; Hancke and Wuttke, 1979) or injection of the alpha-adrenergic blocker, phenoxybenzamine (Ratner, 1971) blocked ovulation and the proestrous LH increase. Injection of the alpha-antagonist dibenamine also inhibited ovulation in rabbits (Sawyer *et al.*, 1947).

Injection of NE into the 3rd cranial ventricle increased plasma LH levels in rabbits (Sawyer *et al.*, 1974; Kreig and Sawyer, 1976) and rats (Kamberi *et al.*, 1970; Vijayan and McCann, 1978). However, NE did not alter LH release from rat pituitaries incubated *in vitro* (Kamberi and McCann, 1969; Quijada *et al.*, 1973). Negro-Vilar *et al.* (1979), Sarkar and Fink (1981) and Ojeda *et al.* (1982) demonstrated that NE stimulated LHRH release from the median eminence via alpha-receptors. Together, these results indicate that NE consistently increases LH release by stimulating LHRH secretion via alpha-receptors.

Dopamine has also been shown to be involved in modulating pulsatile and preovulatory LH releases in mammals (Clemens *et al.*, 1977; Drouva and Gallo, 1977; Negro-Vilar *et al.*, 1982). However, unlike NE, dopamine (DA) has been found to both

stimulate and inhibit LH secretion.

Kamberi *et al.* (1971), and Schneider and McCann (1970) reported that intraventricular injection of DA elevated LH levels in rats. The secretion of LH from rat pituitaries incubated *in vitro* was not affected by direct administration of DA, but was significantly increased by plasma collected from hypothalamo-hypophysial portal vessels of rats injected intraventricularly with DA (Kamberi *et al.*, 1969, 1970). Dopamine has been shown to increase LHRH secretion from median eminence of rats *in vitro* (Rotsztejn *et al.*, 1977; Negro-Vilar *et al.*, 1979). Löfstrom *et al.* (1976) proposed that DA terminals of the tuberoinfundibular pathway synapsed with LHRH-containing neurons in the medial and palisadic zones of the median eminence in an axo-axonal fashion to increase LHRH release.

On the other hand, other evidence suggests that DA inhibits LH release by decreasing LHRH secretion. Injection of metoclopramide, a DA antagonist, increased plasma LH in women (Quigley *et al.*, 1979). Results of studies on DA turnover, plasma LH levels and brain LHRH contents during estrous cycles (Fuxe *et al.*, 1967; Ahren *et al.*, 1971; Demarest *et al.*, 1981; Rance *et al.*, 1981b) or after gonadectomy (Jimenez *et al.*, 1977; Negro-Vilar *et al.*, 1982) indicated that DA decreased LHRH and LH release in rats. Advis *et al.* (1980), Simpkins *et al.* (1980), and Rance *et al.* (1981a) demonstrated that steroid negative feedback on LH release involved DA inhibition of LHRH secretion.

The magnitude of the LHRH and LH surges in rats, induced by treatment with pregnant-mare-serum, was potentiated by two DA antagonists, pimozide and domperidone, but reduced by another DA receptor blocker, haloperidol (Sarkar and Fink, 1981). In mammals, more than one type of DA receptor exists (for review see Kebabian and Calne, 1979; Creese *et al.*, 1981; Creese, 1982); it is possible that the stimulatory and inhibitory DA influences on LH release are mediated by different types of DA receptors.

Although it is generally believed that DA affects LH secretion in mammals by altering LHRH release (see above), DA has also been shown to directly inhibit the actions of LHRH in rabbits (Dailey *et al.*, 1878) and humans (Judd *et al.*, 1978; Huseman *et al.*, 1980).

In certain teleost species, including the goldfish, the gonadotrops are directly innervated by two morphologically distinct types of hypothalamic neurons resembling peptidergic and aminergic nerve fibers, respectively (for review see Peter and Crim, 1979;

Ball 1981). This provides the possibility that gonadotropin (GtH) release can be regulated by hypothalamic peptide hormones as well as by direct actions of neurotransmitters. In teleosts, the presence in the hypothalamus of a GtH-releasing hormone (GnRH) that is similar to LHRH has been demonstrated, and the mammalian LHRH and its superactive analogues have been shown to stimulate GtH secretion (for review see Peter, 1982a, b, 1983). Sherwood *et al.* (1982) reported that the GnRH of chum salmon differs from LHRH by amino acid substitutions at the 7th and 8th positions; the structure of the salmon GnRH is Trp^{7}-Leu^{8}-LHRH. Based on results from brain lesioning experiments, Peter and Paulencu (1980) suggested a GtH release-inhibitory factor (GRIF) to be present in the goldfish.

Relatively few data are available on the possible influences of neurotransmitters on GtH release in fish. Morphologically, the activities of the gonadotrops in the black molly and medaka appeared to be enhanced by reserpine treatments *in vivo* (Egami and Ishii, 1962; Sage and Bromage, 1970). The adenyl cyclase activity of the pars distalis of goldfish, measured *in vitro*, was increased by NE and epinephrine (Deery, 1975). These results suggest that neurotransmitters may influence GtH secretion from gonadotrops in teleosts.

In this thesis, the possible involvement of DA and NE in the regulation of GtH secretion in goldfish, *Carassius auratus*, was investigated by monitoring the changes in serum GtH levels following various pharmacological manipulations, and by studying the influences of DA and NE on GtH release by dispersed pituitary cells and/or pituitary fragments. GtH levels were measured by radioimmunoassay using an antibody generated against a carbohydrate-rich carp GtH purified by B. Breton according to a procedure similar to Idler and Ng (1979; see Peter *et al.*, 1982). According to the classification proposed by Idler, the carbohydrate-rich GtH stimulates oocyte maturation, ovulation, steroidogenesis, cyclic AMP activity and spermiation, whereas the carbohydrate-poor GtH is mainly involved in the stimulation of vitellogenin uptake into oocytes (for review on the number and classification of GtH(s) in teleosts, see Peter 1981, Burzawa-Gerard, 1982a, b; Idler, 1982). Only the carbohydrate-rich GtH can be measured by radioimmunoassay at present; therefore, all references to teleostean GtH in this thesis pertain to this particular GtH.

Experiments contained in this thesis are divided into seven main studies, each presented as a separate chapter. Chapter II describes initial investigations into the possible involvement and influences of catecholaminergic neurotransmitters in the regulation of GtH secretion in female goldfish. The possible role of DA as a GtH release-inhibitor was investigated in experiments described in Chapter III. Chapter IV contains experimental results of a further investigation into the action of DA on spontaneous GtH release from the hetero-transplanted pars distalis of male goldfish. The possible role of DA in the control of the preovulatory GtH release in goldfish was studied in Chapter V. The specificity of the DA inhibition of GtH release was investigated in experiments described in Chapter VI. Results from experiments on the effects of NE and alpha-adrenergic mechanisms on GtH secretion are reported in Chapter VII. In Chapter VIII, observations from *in vitro* experiments investigating the effects of DA and NE on GtH release are presented.

II. **Influence of Catecholamines on Gonadotropin Secretion in Goldfish,** *Carassius auratus.*

(The text of this chapter is in Appendix I, as a reprint from General and Comparative Endocrinology 49, 22–31, 1983.)

III. Effects of Dopamine on Gonadotropin Release in Female Goldfish, *Carassius auratus*.

(The text of this chapter is in Appendix II, as a reprint from Neuroendocrinology 36, 351–357, 1983.)

IV. Effects of Dopamine and Apomorphine on Gonadotropin Release from the Transplanted Pars Distalis in Goldfish.

A. Introduction

Peter *et al.* (1983) reported that transplanting the pars distalis of one goldfish into the cranial cavity beside the brain (juxta position) of a recipient goldfish of matched sexual condition and body weight resulted in increased serum GtH concentrations in the recipient fish; removal of the transplant resulted in the return to normal GtH levels. This demonstrates that the pars distalis of goldfish releases GtH spontaneously following disconnection from the hypothalamus. Transplantation of the pars distalis into the brain ventricles in the preoptic region or under the optic tectum resulted in lower serum GtH levels in the recipient fish than juxta transplantation, indicating the presence of a GRIF in the brain.

Goldfish bearing a pars distalis transplant in the juxta position may be a useful *in vivo* model for studying the direct action of neurotransmitters on spontaneous GtH release. Results from Chapter III demonstrated that DA has GRIF activity to inhibit GtH secretion and to block the actions of GnRH. In the present investigation, the direct action of DA on GtH release was further studied by monitoring the GtH concentrations in male goldfish bearing pars distalis transplants in the juxta position before and at various times following intraperitoneal injection of dopamine or apomorphine.

B. Materials and Methods

General

The procedures described in Chapter II for photoperiod–temperature regime, fish maintenance, handling, and blood sampling were followed. Apomorphine and DA injection solutions were as described in Chapter III. Control fish were injected with an equivalent volume of the drug vehicle (vehicle).

Only sexually mature spermiating males were used in this study. Partes distalis from donor male goldfish were transplanted into the cranial cavity (juxta position) of recipient male golfish of matched body weight as described by Peter *et al.* (1983). Serum

GtH concentrations were measured by radioimmunoassay (Peter *et al.*, 1983). The fish were killed by decapitation after each experiment.

Signed–ranks test was used to compare preinjection and postinjection GtH concentrations within experimental groups. Mann–Whitney U test was used to compare GtH values between experimental and control GtH groups (Snedecor and Cochran, 1971).

Experimental Treatments

DA (10 and 100 ug/g body weight) or vehicle was injected intraperitoneally into male goldfish bearing pars distalis transplants in the juxta position (recipient fish) at day 2 after the transplant operation. Blood samples were taken just before, and at 2 and 6 h after DA or vehicle injection.

Apomorphine (10 and 20 ug/g body weight) or vehicle was injected intraperitoneally into recipient fish at day 1 after the transplant operation. Blood samples were collected just prior to, and at 6 and 24 h after apomorphine or vehicle injection.

C. Results

Male goldfish bearing pars distalis transplants in the juxta position (recipient fish) had significantly higher serum GtH concentrations than sham–operated fish without a transplant at one and two days following the transplant operation (Tables 4.1 and 4.2). Characteristic of fish bearing pars distalis transplants, serum GtH concentrations in the recipient fish of this study were highly variable; therefore, the serum GtH values at various times after drug or vehicle injection were expressed as a percent of the preinjection values. DA at either 10 or 100 ug/g significantly decreased serum GtH levels in recipient fish at 2 h post–injection compared to control and preinjection values (Table 4.1). At 6 h after injection, serum GtH levels in recipient fish injected with DA at 100 ug/g or the DA–vehicle were significantly higher than preinjection values (Table 4.1). Serum GtH levels in recipient fish injected with DA at 10 ug/g were significantly lower than vehicle injected control at 6 h postinjection (Table 4.1). At 6 h following apomorphine or vehicle injection, serum GtH levels in recipient fish injected with the vehicle were significantly higher than preinjection values (Table 4.2). Intraperitoneal injection of apomorphine at 10 ug/g significantly decreased serum GtH levels in recipient fish at 6 h postinjection compared to

Table 4.1. Effects of intraperitoneal injection of DA on serum GtH concentrations in male goldfish bearing a pars distalis transplant in the juxta position[1,2]. GtH levels are given as mean±SE.

Treatment		GtH (% preinjection) at h postinjection	
	n	2	6
vehicle	9	103.7±5.1	155.8±14.4[3]
DA, 10 ug/g	9	86.9±3.0[4,5]	111.5±7.0[6]
DA, 100 ug/g	9	84.5±3.2[4,6]	125.9±3.2[3]

1 This experiment was performed in late May. Body weight (mean±SE) of donor and recipient fishes were 29.0±1.4 and 30.6±1.5 g, respectively.

2 At the time of vehicle or DA injection serum GtH concentrations in recipient fish (33.6±2.1 ng/ml; mean±SE, n=27) were significantly higher ($P<0.001$) than those (1.4±0.2 ng/ml; n=8) of sham-operated fish without a transplant.

3 Significantly higher than preinjection, $P<0.025$.

4 Significantly less than preinjection, $P<0.005$.

5 Significantly less than values in vehicle injected controls at the same sample time, $P<0.025$.

6 Significantly less than values in vehicle injected controls at the same sample time, $P<0.01$.

Table 4.2. Effects of intraperitoneal injection of apomorphine on serum GtH concentrations in male goldfish a bearing pars distalis transplant in the juxta position[1,2]. GtH levels are given as mean±SE.

Treatment		GtH (% preinjection) at h postinjection	
	n	6	24
vehicle	6	179±45.8[3]	113.7±30.9
apomorphine, 10 ug/g	7	94.5±7.8[4]	69.9±24.3[5]
apomorphine, 20 ug/g	6	128.1±23.2	61.0±14.4[6]

1 This experiment was performed in February. Body weight (mean±SE) of donor and recipient fishes were 33.3±1.8 and 36.2±1.6 g, respectively.

2 At the time of vehicle or apomorphine injection, recipient fish had serum GtH concentrations (78.3±12.1 ng/ml; mean±SE, n=19) that were significantly higher ($P<0.001$) than those (1.9±0.4 ng/ml; n=11) of sham-operated fish without a transplant.

3 Significantly greater than preinjection value, $P<0.01$.

4 Significantly less than values in vehicle injected control at the same sample time, $P<0.025$.

5 Significantly less than preinjection and control values, $P<0.025$.

6 Significantly less than preinjection and control values, $P<0.05$.

controls (Table 4.2). At 24 h after injection, serum GtH concentrations in recipient fish injected with apomorphine at either 10 or 20 ug/g were significantly lower than control and preinjection values (Table 4.2).

D. Discussion

Transplantation of the pars distalis from one goldfish to beside the brain of another goldfish ("juxta" position) resulted in increased serum GtH concentrations in the recipient fish, as in previous studies (Peter *et al.*, 1983). As reviewed in the Introduction, changes in the serum GtH levels of fish bearing pars distalis transplants reflect changes in the GtH secretion rate of the transplanted pars distalis. However, the serum GtH concentrations in the recipient fish of this study were lower than those reported earlier for sexually mature males bearing a juxta pars distalis transplant (Peter *et al.*, 1983). The differences between the serum GtH levels in the recipient fish of the two studies may be the result of seasonality. Experiments in the present investigation utilized male goldfish prior to (February, apomorphine experiment) or at the end of (late May, dopamine experiment) the normal spawning season, whereas the mature male goldfish used by Peter *et al.* (1983) were at the time of the normal spawning season (April). Peter *et al.* (1983) demonstrated seasonal differences in the GtH released by pars distalis transplants, with transplants using sexually regressed females, or early recrudescing males, releasing less GtH than transplants using recrudescing or mature females, and mature males, respectively. Also, in male goldfish, the GtH release–response to injections of a superactive analogue of the mammalian luteinizing hormone–releasing hormone is lower at the end than at the start of the spawning season (Lin *et al.*, 1982), providing further evidence for seasonal differences in the ability of the goldfish pituitary to release GtH.

In this study,intraperitoneal injection of DA and its agonist, apomorphine, consistently decreased serum GtH levels in recipient fish bearing pars distalis transplants at 2 and 24 h postinjection, respectively (Tables 4.1 and 4.2). Although the transplants were located within the cranial cavity, they were outside the blood–brain barrier, and were therefore exposed to the intraperitoneally applied drug solutions. These results suggest that DA directly inhibited the spontaneous release of GtH from the "juxta" pars distalis transplants. Intraperitoneal injection of DA or apomorphine has been shown to inhibit

spontaneous GtH release from pituitaries of female goldfish lesioned in the preoptic area to abolish the inhibitory hypothalmic influence on GtH secretion (Chapter III). When viewed together, these observations indicate that DA may act directly on gonadotrops to inhibit spontaneous GtH release in female as well as in male goldfish.

In summary, results of this study are consistent with the idea that DA acts as a GRIF.

V. Effects of Pimozide and LHRH-A on Serum Gonadotropin Concentrations, Germinal Vesicle Migration and Ovulation in Female Goldfish.

A. Introduction

As reviewed in the General Introduction (Chapter I), GtH release in teleosts is under the influence of GnRH and an unidentified GRIF. Spontaneous ovulation in goldfish is induced and preceded by a surge in serum GtH levels (Stacey *et al.*, 1979). The preovulatory surge of GtH may be regulated by a stimulation of GtH release by GnRH and/or a release from inhibition by GRIF (Peter and Paulencu, 1980). Results from Chapters III and IV suggest that DA has GtH release-inhibitory activity in goldfish, by actions directly on gonadotrops to inhibit spontaneous GtH release, and by blocking the actions of GnRH. In this chapter, the possible role of DA in the neuroendocrine regulation of the preovulatory GtH release was investigated by studying the effects of intraperitoneal injections of the DA antagonist, pimozide, on serum GtH concentrations, germinal vesicle migration, and ovulation in female goldfish injected with a superactive LHRH analogue, LHRH-A.

B. Materials and Methods

The procedures described in Chapter II for photoperiod-temperature regime, fish maintenance, handling and blood sampling were followed. Serum GtH concentrations were measured by radioimmunoassay as described in Chapter IV. Only gravid females (as indicated by a soft, distended abdomen; Stacey *et al.*, 1979) were used. Floating artifical plants (bundles of 10-15 cm strands of green acrylic fibre) were added to the tanks at the begining of each experiment as visual stimuli for ovulation (Yamazaki, 1965; Stacey *et al.*, 1979).

LHRH-A (Sigma), dissolved in fish physiological saline (PS) as described in Chapter III, was injected intraperitoneally (0.1 ug/g body weight) either as a single injection or as two injections 12 h apart (Table 5.1). Pimozide, prepared as described in Chapter III, was injected intraperitoneally as a suspension at a dose of 10 ug/g body weight. Control groups were given an equivalent volume of PS and/or the pimozide vehicle (vehicle).

Table 5.1. Summary of treatments of gravid female goldfish, held at 12°C, in various experimental groups.[1]

Experiments	Injections		Sampling times
	1st (21:00; −12 h)	2nd (09:00; 0 h)	
5.1[2]	PS	PS+vehicle	Blood sampled at 0,
	PS	PS+PIM	6, & 24 h. Germinal
	LHRH−A	LHRH−A+PIM	vesicle migration
	LHRH−A	LHRH−A+vehicle	checked at 24 h.
	uninjected	uninjected	
5.2	PS	PS+vehicle	Blood sampled at 0, 24 &
	LHRH−A	LHRH−A+PIM	72 h. Ovulation checked
	LHRH−A	LHRH−A+vehicle	at 0, 24, 48 & 72 h.
5.3	PS+vehicle	PS	Blood sampled at 0,
	PS+PIM	PS	24 & 48 h. Ovulation
	LHRH−A+PIM	LHRH−A	checked at 0, 24
	LHRH−A+vehicle	LHRH−A	& 48 h.
	PS+PIM	LHRH−A	
	PS+vehicle	LHRH−A	
5.4	uninjected	PS+vehicle	Blood sampled at 24,
	uninjected	PS+PIM	48 & 72 h. Ovulation
	uninjected	LHRH−A+PIM	checked at 24,
	uninjected	LHRH−A+vehicle	48 & 72 h.

[1] Abbreviations used: LHRH−A, des Gly[10], [D−Ala[6]] luteinizing hormone−releasing hormone ethylamide; PIM, pimozide; PS, fish physiological saline.

[2] Floating artificial plants were present throughout the entire acclimation and experimental period for experiment 5.1.

Ovulation was indicated by the release of a stream of ripened translucent oocytes from the ovipore following application of a slight pressure to the abdomen (Yamazaki, 1965; Stacey *et al.*, 1979). Germinal vesicle migration was checked at the termination of one experiment by examining the location of the germinal vesicle in oocytes that had been cleared for 15–20 minutes in a solution of ethanol:formalin:glacial acetic acid (6:3:1; by volume). Germinal vesicle migration was considered to have occurred when the germinal vesicle in the majority of the oocytes was located just below the animal pole (Yamazaki, 1965).

Serum GtH data were log–transformed and multiple comparisons between different treatment groups at each sampling time made using one–way analysis of variance and Duncan's multiple range test. Chi–square test was used to compare the number of fish containing ovulated oocytes or oocytes that had undergone germinal vesicle migration between treatment groups (Snedecor and Cochran, 1971).

The details of treatments in various experimental groups are summarized in Table 5.1.

C. Results

Experiment 5.1. Effects of pimozide injected at the time of the second LHRH-A injection on serum GtH concentrations and germinal vesicle migration in gravid female goldfish held at 12°C.

The serum GtH concentrations in uninjected fish were not different from those of the PS + PS+vehicle injected fish at any sample time (Table 5.2). Serum GtH concentrations in the PS + PS+pimozide injected fish were significantly higher than levels in the PS + PS+vehicle injected and uninjected fish at 24 h after the second injection (Table 5.2). At the time of the second LHRH–A injection, fish receiving LHRH–A in the first injection had significantly higher serum GtH concentrations than those injected previously with PS (Table 5.2). At 6 h after the second LHRH–A injection, serum GtH concentrations in fish injected with either LHRH–A+pimozide or LHRH–A+vehicle at the second injection were not significantly different from one another, but the serum GtH levels in both groups were significantly higher than levels in the PS + PS+pimozide injected, PS + PS+vehicle injected,

Table 5.2. Effects of pimozide (PIM) injected at the time of the second LHRH-A injection on serum GtH concentrations and germinal vesicle migration in gravid female goldfish (GSI=12.7±0.5%) held at 12°C. At each individual sampling time, serum GtH values (mean±SE) that are similar (P>0.05) are identified by similar superscripts.

1st injection (−12 h)	2nd injection (0 h)	n	serum GtH, ng/ml			germinal vesicle migration
			0 h	6 h	24 h	
uninjected	uninjected	9	3.6±0.8[1]	3.0±0.4[1]	3.1±0.4[1]	0
PS	PS+vehicle	9	2.8±0.5[1]	3.0±0.8[1]	2.6±0.3[1]	0
PS	PS+PIM	8	3.6±0.4[1]	5.1±1.2[1]	6.2±1.4[2]	0
LHRH-A	LHRH-A+PIM	9	16.0±4.8[2]	113.4±59.3[2]	82.7±17.5[3]	5▲
LHRH-A	LHRH-A+vehicle	9	18.3±4.6[2]	33.4±12.6[2]	13.7±6.1[2]	1

▲ Significantly greater than all other groups (P<0.005).

and the uninjected groups (Table 5.2). At 24 h after the second LHRH-A injection, serum GtH concentrations in the LHRH-A + LHRH-A+vehicle injected fish were not different from levels in the PS + PS+pimozide injected fish, but were significantly higher than levels in the uninjected and PS + PS+vehicle injected fish (Table 5.2). The LHRH-A + LHRH-A+pimozide injected fish had significantly higher serum GtH levels than all other groups at 24 h after the second injection (Table 5.2). The number of fish bearing oocytes that showed germinal vesicle migration was significantly higher in the LHRH-A + LHRH-A+pimozide injected group than in any other treatment group (Table 5.2).

Experiment 5.2. Effects of pimozide injected at the time of the second LHRH-A injection on serum GtH concentrations and ovulation in gravid female goldfish held at 12°C.

At the time of the second LHRH-A injection, serum GtH concentrations in fish injected 12 h earlier with LHRH-A were significantly higher than those in fish injected previously with PS (Table 5.3). The serum GtH concentrations in the LHRH-A + LHRH-A+vehicle injected fish were significantly higher than the levels in the PS + PS+vehicle injected fish at 24 but not at 72 h after the second injection (Table 5.3). The serum GtH concentrations in the LHRH-A + LHRH-A+pimozide injected fish were significantly higher than the PS + PS+vehicle and LHRH-A + LHRH-A+vehicle injected fish at both 24 and 72 h after the second injection (Table 5.3). The presence of ovulated fish was first detected at 48 h after the second injection; no additional ovulated fish were found at 72 h after the second injection (Table 5.3). The number of ovulated fish was significantly higher in the LHRH-A + LHRH-A+pimozide injected group than in the LHRH-A + LHRH-A+vehicle and the PS + PS+vehicle injected groups (Table 5.3).

Experiment 5.3. Effects of pimozide injected with, or in place of, the first of two LHRH-A injections on serum GtH concentrations and ovulation in gravid female goldfish held at 12°C.

At the time of the second injection, serum GtH concentrations in fish injected 12 h earlier with PS+vehicle were not different from those in fish injected previously with PS+pimozide (Table 5.4). At the time of the second injection, serum GtH concentrations in

Table 5.3. Effects of pimozide (PIM) injected at the time of the second LHRH-A injection on serum GtH concentrations and ovulation in gravid female goldfish (GSI= 14.9±0.7%) held at 12°C. At each individual sampling time, serum GtH values (mean±SE) that are similar to each other (P>0.05) are identified by similar superscripts.

1st injection	2nd injection	n	serum GtH, ng/ml			fish	ovulated
(-12 h)	(0 h)		0 h	24 h	72 h	48 h	72 h
PS	PS+vehicle	8	4.8±0.8[1]	7.3±1.4[1]	4.5±0.7[1]	0	0
LHRH-A	LHRH-A+PIM	9	61.2±20.1[2]	294.8±51.8[2]	129.9±45.1[2]	8▲	8▲
LHRH-A	LHRH-A+vehicle	9	56.2±21.0[2]	65.8±19.3[3]	11.7±6.6[1]	2□	2□

▲ Significantly greater than all other groups (P<0.005).

□ Not significantly different from control group.

Table 5.4. Effects of pimozide (PIM) injected with, or in place of, the first of two LHRH-A injections on serum GtH concentrations and ovulation in gravid female goldfish (GSI=15.0±0.5%) held at 12°C. At each individual sampling time, serum GtH values (mean±SE) that are similar to each other (P>0.05) are identified by similar superscripts.

1st injection	2nd injection	n	serum GtH, ng/ml			fish	ovulated
(−12 h)	(0 h)		0 h	24 h	48 h	24 h	48 h
PS+vehicle	PS	7	5.0±1.0[1]	6.1±1.0[1]	5.6±0.9[1]	0	0
PS+PIM	PS	7	12.7±5.4[1]	68.8±41.7[2]	54.4±37.7[2]	0	1
LHRH-A+PIM	LHRH-A	8	205.2±86.7[2]	192.7±61.2[3]	143.3±61.5[2]	3△	7▲
LHRH-A+vehicle	LHRH-A	8	61.5±21.4[2]	181.9±61.5[3]	83.1±24.5[2]	0	0
PS+PIM	LHRH-A	7	8.4±1.6[1]	217.4±61.7[3]	47.9±17.4[2]	1	5▲
PS+vehicle	LHRH-A	6	6.4±1.2[1]	51.7±24.7[2]	17.5±4.4[2]	0	0

△ Significantly greater than the PS+vehicle + PS, PS+PIM + PS, LHRH-A+vehicle + LHRH-A, and PS+vehicle + LHRH-A injected groups (P<0.05).

▲ Significantly greater than the PS+vehicle + PS, PS+PIM + PS, LHRH-A+vehicle + LHRH-A, and PS+vehicle + LHRH-A injected groups (P<0.005).

fish injected previously with LHRH–A+pimozide were not significantly different from those in fish injected previously with LHRH–A+vehicle; both of the LHRH–A injected groups had significantly higher serum GtH concentrations than all other groups (Table 5.4). At 24 h after the second injection, serum GtH concentrations in the PS+vehicle + LHRH–A and PS+pimozide + PS injected groups were not different from each other, but were significantly higher than those in the PS+vehicle + PS injected group (Table 5.4). At 24 h after the second injection, the LHRH–A+vehicle + LHRH–A, LHRH–A+pimozide + LHRH–A and PS+pimozide + LHRH–A groups had serum GtH concentrations that were not different from one another, but were significantly higher than those of the other treatment groups (Table 5.4). At 48 h after the second injection, serum GtH values in the PS+pimozide + PS, LHRH–A+pimozide + LHRH–A, LHRH–A+vehicle + LHRH–A, PS+pimozide + LHRH–A, and PS+vehicle + LHRH–A injected groups were similar, but all were significantly higher than the PS+vehicle + PS injected group (Table 5.4).

Four fish ovulated by 24 h after the second injection and an additional 9 fish ovulated between 24 and 48 h after the second injection (Table 5.4). At 24 h after the second injection, the number of ovulated fish in the LHRH–A+pimozide + LHRH–A injected group was significantly higher than that in the PS+vehicle + PS, PS+pimozide + PS, LHRH–A+vehicle + LHRH–A and PS+vehicle + LHRH–A injected groups, but not different from that in the PS+pimozide + LHRH–A injected group (Table 5.4). At 48 h after the second injection, the number of ovulated fish in the LHRH–A+pimozide + LHRH–A and PS+pimozide + LHRH–A groups were not different from each other, but were significantly higher than that of the other treatment groups (Table 5.4).

Experiment 5.4: Effects of a single injection of pimozide and/or LHRH-A on serum GtH concentrations and ovulation in gravid female goldfish held at 12°C.

The serum GtH concentrations in fish injected with PS+pimozide were significantly higher than those in fish injected with PS+vehicle at 24, but not at 48 and 72 h post–injection (Table 5.5). Serum GtH concentrations in the LHRH–A+pimozide injected fish were not significantly different from those in fish injected with LHRH–A+vehicle, but were significantly higher than those in fish injected with PS+vehicle at all times (Table 5.5). Serum GtH concentrations in fish injected with LHRH–A+vehicle were significantly higher

Table 5.5. Effects of a single injection of pimozide (PIM) and/or LHRH-A on serum GtH concentrations and ovulation in gravid female goldfish (GSI= 19.7±0.8%) held at 12°C. At each individual sampling time, serum GtH values (mean±SE) that are similar to each other (P>0.05) are identified by similar superscripts.

injection	n	serum GtH, ng/ml			fish	ovulated
(0 h)		24 h	48 h	72 h	48 h	72 h
PS+vehicle	8	7.1±1.6[1]	5.9±1.9[1]	3.9±0.6[1]	0	0
PS+PIM	8	15.0±4.3[2]	7.6±1.6[1,2]	4.7±0.7[1,2]	0	0
LHRH-A+PIM	8	89.2±28.4[3]	54.9±23.8[3]	7.4±1.7[2]	2	3△
LHRH-A+vehicle	8	72.0±29.8[3]	51.1±40.4[2,3]	4.7±0.6[1,2]	0	0

△ Significantly greater than all other groups (P<0.05).

than those in fish injected with PS+vehicle at 24 and 48, but not at 72 h post-injection (Table 5.5). Serum GtH concentrations in the PS+pimozide injected fish were significantly lower than those in the LHRH-A+pimozide injected fish at 24 and 48, but not at 72 h post-injection (Table 5.5). Ovulation was detected only in the LHRH-A+pimozide injected fish, and by 72 h post-injection, the number of ovulated fish in this LHRH-A injected group was significantly higher than that of the other treatment groups (Table 5.5).

D. Discussion

Injection of the DA antagonist, pimozide, increased serum GtH concentrations in gravid female goldfish (Tables 5.2, 5.4 and 5.5), similar to a previous study using female goldfish in early stages of ovarian recrudescence (Chapter III). These results are consistent with the conclusion that DA has GtH release inhibitory activity in goldfish (Chapter III). To study the possible involvement of DA in the regulation of the preovulatory GtH surge, the effects of pimozide on serum GtH concentrations, germinal vesicle migration and ovulation in LHRH-A injected female goldfish were studied.

Similar to previous experiments using male goldfish (Peter, 1980), LHRH-A, given as a single or two intraperitoneal injections 12 h apart, was effective in increasing serum GtH concentrations in gravid female goldfish (Tables 5.2, 5.3, 5.4 and 5.5). Serum GtH concentrations in female goldfish given two injections of LHRH-A at a 12 h interval were significantly higher than those in fish given an injection of PS 12 h before a single LHRH-A injection (Table 5.4). This suggests that the LHRH-A stimulated GtH-release response can be potentiated by a previous injection of the releasing hormone in female as well as in male goldfish (Peter, 1980).

In teleosts, germinal vesicle migration is a preliminary step to ovulation (Yamazaki, 1965; Billard *et al.*, 1978). Final oocyte maturation and ovulation in goldfish is induced by a preovulatory GtH surge (for review see Peter, 1981). LHRH-A stimulated GtH release but did not significantly induce ovulation nor germinal vesicle migration in gravid female goldfish in this study (Tables 5.2, 5.3, 5.4 and 5.5). Although Lam *et al.* (1975, 1976) reported that multiple injections of very high dosages of LHRH induced ovulation in goldfish, and LHRH-A has been used to induce ovulation in several teleost species (for review see Lam, 1982; Lin, 1982; Peter, 1982a), Peter (unpublished results) has not

successfully induced ovulation in a high proportion (>50%) of goldfish using either single or multiple injections of various doses of LHRH or LHRH-A. Injection of pimozide together with the second LHRH-A injection not only significantly potentiated and prolonged the LHRH-A stimulated release of GtH, but also increased the serum GtH to levels similar to those found in spontaneously ovulating goldfish (168±14 ng/ml, n=26; Stacey *et al.*, 1979). In addition, the injection of LHRH-A and pimozide significantly increased the frequency of ovulation (Tables 5.3, 5.4 and 5.5). The occurrence of ovulation in these pimozide and LHRH-A treated fish was preceded 24 h earlier by germinal vesicle migration (Table 5.2). Pimozide injections that were not accompanied by LHRH-A injection did not increase serum GtH levels to those found in ovulating goldfish and did not significantly induce germinal vesicle migration or ovulation (Tables 5.2, 5.4 and 5.5). These results suggest that the preovulatory GtH surge in the goldfish is normally regulated by both a stimulation of GtH release by GnRH and the removal of the DA inhibition on GtH release. The involvement of a GRIF in the regulation of the preovulatory GtH surge in goldfish has been suggested previously (Peter and Paulencu, 1980).

To examine the interactions between GnRH and DA in the regulation of the preovulatory GtH surge and ovulation, pimozide was injected together with the first (priming) injection of LHRH-A or in place of the priming injection of LHRH-A (Experiment 5.3), or together with a single injection of LHRH-A (Experiment 5.4). Pimozide injected with or instead of the priming LHRH-A injection was equipotent ($P>0.05$, Chi-square) in inducing ovulation as pimozide injected with the second of two LHRH-A injections (Tables 5.3 and 5.4). However, pimozide injected together with a single injection of LHRH-A was not as effective ($P<0.05$, Chi-square) at inducing ovulation as pimozide injected with the first or the second of two LHRH-A injections (compare Table 5.5 to Tables 5.3 and 5.4). These results indicate that a priming injection of LHRH-A will increase the frequency of induced ovulation, but that it is not a necessity for inducing ovulation when the DA inhibition on GtH release is suppressed. The results also indicate that prior release from DA inhibition greatly potentiates the response to LHRH-A.

Twenty four h before ovulation was first detected, serum GtH concentrations in fish injected with a combination of pimozide and LHRH-A were generally higher than those in their respective LHRH-A injected control fish (Tables 5.2, 5.3, 5.4 and 5.5); however, the

differences in serum GtH levels were not always significant in cases where the induced ovulation was not well synchronized (Tables 5.4 and 5.5). The relatively low mean serum GtH levels in fish injected with pimozide together with a single injection of LHRH-A might reflect the lower efficiency of this treatment in inducing ovulation (Table 5.5). In Experiment 5.3, serum GtH concentrations in fish injected with 2 injections of LHRH-A were in the range of those found in spontaneously ovulating fish at 24 h after the second injection, although none of the fish in this group ovulated (Table 5.4). The preovulatory GtH surge in spontaneously ovulating goldfish is in the form of a rapid increase in serum GtH concentration confined to an interval of approximately 12 h (Stacey *et al.*, 1979). Perhaps the rate of increase in blood GtH concentration may be as important as the absolute serum GtH concentration in inducing ovulation in goldfish. It is likely that release of the DA inhibition on GtH release allows the gonadotrops to rapidly increase GtH secretion in response to the releasing hormone, producing a rapid increase in serum GtH levels. Further experiments are required to study the importance of the rate of increase in blood GtH in inducing ovulation in goldfish.

The dose of LHRH-A used in this study was about 10 times higher than that used in inducing ovulation in various salmonids and domesticated carp species (for review see Lam, 1982). Experiments are in progress to determine if smaller doses of pimozide and LHRH-A can be used to effectively stimulate GtH release and induce ovulation in the goldfish. Results from preliminary experiments have already shown that smaller doses of pimozide and LHRH-A can be used to effectively stimulate GtH release and induce ovulation in the goldfish (M. Sokolowska *et al.*, unpublished results).

In summary, pimozide potentiates the effects of LHRH-A on serum GtH concentrations and increases the proportion of fish in which ovulation can be induced by LHRH-A. Blockage of the DA inhibition on GtH release by pimozide allows for a rapid increase in GtH release in response to LHRH-A, which may be important for induction of oocyte maturation and ovulation in goldfish. The present study suggests that the normal preovulatory GtH surge in goldfish is regulated by both a removal of an inhibition on GtH release and a stimulation of GtH secretion by GnRH.

VI. Effects of Catecholaminergic Agonists and Antagonists on Serum Gonadotropin Concentrations and Ovulation in Goldfish: Evidence for Specificity of Dopamine Inhibition of Gonadotropin Secretion.

A. Introduction

Results from *in vivo* experiments on goldfish (Chapters III and IV) demonstrate that DA and its agonist, apomorphine, inhibit spontaneous GtH release and release stimulated by a superactive analogue of LHRH. Removal of the DA inhibition on GtH secretion may be part of the mechanism regulating the ovulatory GtH surge in some teleost fishes; injection of the DA antagonist, pimozide, significantly potentiated the ability of LHRH analogues to stimulate GtH release and ovulation in female goldfish (Chapter V). In Chapter III, it was proposed that DA may inhibit GtH release by interfering with the formation of adenosine cyclic monophosphate (cAMP). In mammals, the so-called D-2 DA receptors have been shown to be negatively coupled to adenyl cyclase and have been identified in the anterior pituitary (for review see Creese *et al.*, 1981; Creese, 1982).

In mammals, DA also has some affinity for alpha- and beta-adrenergic receptors; therefore, it remains possible that DA inhibits GtH release in goldfish via nonspecific actions on adrenergic receptors. In this study, the possible involvement of D-2 - like DA receptors in mediating the DA inhibition of GtH release in goldfish, as well as the specificity of this inhibitory DA influence were further investigated by monitoring the effects of intraperitoneal injections of DA agonists (apomorphine and bromocriptine) and antagonists (pimozide and metoclopramide), an alpha-adrenergic antagonist (phentolamine), a beta-adrenergic antagonist (propranolol), and an alpha-adrenergic sympathomimetic substance (octopamine) on serum GtH levels and/or ovulation in normal fish or fish injected with a LHRH analogue.

B. Materials and Methods

General

Goldfish, common or comet variety, purchased from Ozark Fisheries, Stoutland, Missouri, were held on a photoperiod–temperature regime as described in Chapter II. The procedures for fish maintenance, handling, and blood sampling were outlined in Chapter II. Serum GtH concentrations were measured by radioimmunoassay as described in Chapter IV.

Octopamine HCl (octopamine), 2–bromo–alpha–ergocryptine methane sulfonate (bromocriptine), and propranolol HCl (propranolol) were purchased from Sigma, St. Louis, Missouri. Phentolamine HCl (Rogitine, phentolamine) was a gift from Ciba–Geigy Canada Ltd., Dorval, Quebec. LHRH–A was dissolved in fish physiological saline as described in Chapter III. Apomorphine, pimozide and all other drugs listed above were made up in a vehicle of acidified 0.7% saline with 0.1% sodium metabisulfite. All drugs were injected intraperitoneally on a ug/g body weight basis (dosages given below). As in Chapter III, pimozide was injected as a fine suspension, while all other drugs were injected as a solution. Control groups were given an equivalent volume of vehicle and/or PS.

Mann–Whitney U test was used to compare GtH concentrations between the experimental and control groups. For multiple comparisons between treatment groups, serum GtH data were log–transformed prior to testing with one–way analysis of variance and Duncan's multiple range test. Chi–square test was used to compare the number of ovulated fish between treatment groups (Snedecor and Cochran, 1971).

Experiment 6.1. Effects of apomorphine, injected with either the first or the second of two LHRH-A injections, on serum GtH concentrations.

Female goldfish undergoing ovarian recrudescence (GSI=11.1±0.6%; mean±SE; March) were used. LHRH–A (0.1 ug/g) was injected twice at a 12–h interval (first injection at 21:00). Apomorphine (20 ug/g) was injected with the first or the second LHRH–A injection. Blood samples were taken serially at the time of, and at 6 and 24 h after the second LHRH–A injection.

Experiment 6.2. Effects of bromocriptine, injected with both the first and the second of two LHRH-A injections, on serum GtH concentrations.

Spermiating male goldfish (GSI=3.6±0.2%; February) were used. LHRH-A (0.1 ug/g) was injected twice with a 3-h interval (first injection at 09:00). Bromocriptine (2 and 20 ug/g) was injected simultaneously with both of the LHRH-A injections. Blood samples were taken serially at 3, 6 and 24 h after the second LHRH-A injection.

Experiment 6.3. Effects of pimozide, phentolamine, or propranolol on serum GtH concentrations.

Pimozide, phentolamine or propranolol was injected at a dose of 10 ug/g into goldfish (mixed sex) undergoing early stages of gonadal recrudescence (female GSI=3.0±0.2%, n=20; male GSI=2.9±0.2%, n=20; November). Blood samples were taken serially at the time of and at 6 and 24 h following drug injection.

Experiment 6.4. Effects of pimozide, phentolamine or propranolol, injected at the time of the second of two LHRH-A injections, on serum GtH concentrations.

Goldfish (mixed sex) undergoing gonadal recrudescence were used (female GSI=3.6±0.3%, n=37; male GSI=2.4±0.2%, n=22; January). LHRH-A (0.1 ug/g) was injected twice at an interval of 12 h. Pimozide, phentolamine or propranolol were injected at a dose of 10 ug/g at the time of the second LHRH-A injection. All fish were blood sampled at 24 h after the second LHRH-A injection.

Experiment 6.5. Effects of pimozide, metoclopramide or octopamine, injected at the time of the second of two LHRH-A injections, on serum GtH concentrations and ovulation.

Gravid female goldfish, as indicated by a soft, distended abdomen (Stacey *et al.*, 1979) were used (GSI=14.3±1.9%; March). LHRH-A (0.1 ug/g) was injected twice at a 12-h interval. Pimozide (1 and 10 ug/g), metoclopramide (1 and 10 ug/g) or octopamine (10 ug/g) was injected at the time of the second LHRH-A injection. Blood samples were taken at the time of and at 6, 24 and 48 h after the second LHRH-A injection. The occurrence of ovulation, as indicated by the release of a stream of ripened translucent

oocytes from the ovipore following application of a slight pressure to the abdomen (Yamazaki, 1965; Stacey *et al*., 1979), was checked at each sampling time.

C. Results

Experiment 6.1. Effects of apomorphine, injected with either the first or the second of two LHRH-A injections, on serum GtH concentrations.

Fish given two injections of LHRH–A at an interval of 12–h had significantly higher serum GtH concentrations than the PS injected groups at 0, 6 and 24 h after the second injection (Figure 6.1). Injection of apomorphine together with PS did not alter serum GtH concentrations at any sampling time compared to the PS+vehicle controls (Figure 6.1). Fish given apomorphine together with the first LHRH–A injection had significantly lower serum GtH values than the LHRH–A+vehicle injected group at 6 and 24 h after the second LHRH–A injection. Fish receiving apomorphine together with the second LHRH–A injection had significantly lower serum GtH levels than the LHRH–A+vehicle group at 24 h after the last injection. Serum GtH concentrations in fish receiving apomorphine with either the first or the second LHRH–A injection were not different from each other at any sampling time (Figure 6.1).

Experiment 6.2. Effects of bromocriptine, injected with both the first and the second of two LHRH-A injections, on serum GtH concentrations.

Two injections of LHRH–A at a 3–h interval significantlty increased serum GtH levels compared to PS injected fish at all sampling times (Table 6.1). Fish injected simultanesously with LHRH–A and bromocriptine at 20 ug/g had significantly lower serum GtH levels than those injected with LHRH–A and vehicle at 3 h after the second LHRH–A injection. Serum GtH concentrations in fish injected simultaneously with LHRH–A and bromocriptine at 2 ug/g were not significantly different, at any sampling time, from those in LHRH–A injected fish receiving either bromocriptine at 20 ug/g or vehicle. In comparison with the PS+vehicle group, injection of either dosage of bromocriptine together with PS did not alter serum GtH concentrations.

Figure 6.1. Effects of intraperitoneal injection (ip) of apomorphine (20 ug/g; APO) on serum GtH concentrations in LHRH-A injected female goldfish undergoing ovarian recrudescence. LHRH-A (0.1 ug/g) was injected twice at an interval of 12 h. APO was injected at the time of the first or the second LHRH-A injection. Serum GtH levels were plotted as mean±SE. At each sampling time, serum GtH concentrations of groups underlined by the same line are similar (P>0.05).

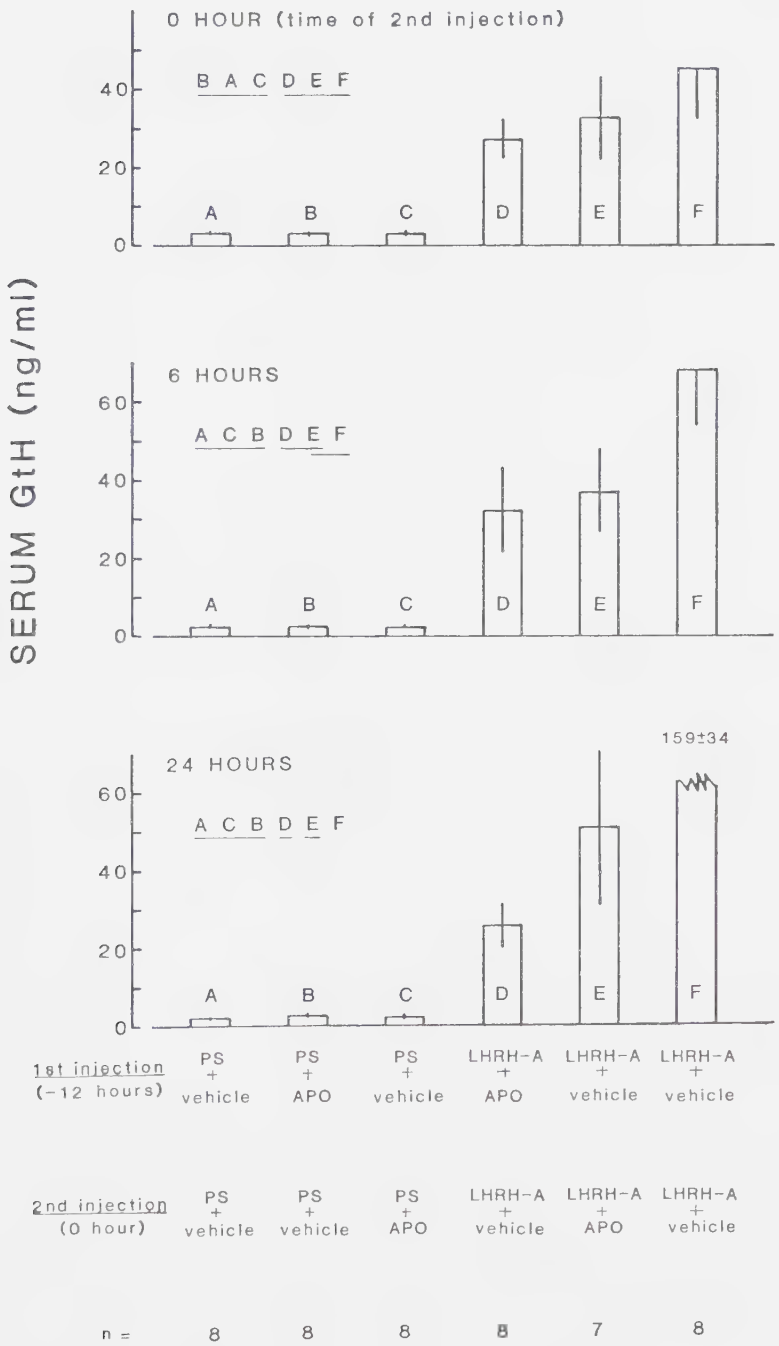


Table 6.1. Effects of intraperitoneal injection (ip) of bromocriptine (2 and 20 ug/g; bromo(2) and bromo(20)), injected simultaneously with LHRH-A (0.1 ug/g, 2 ip injections, 3 h apart), on serum GtH concentrations in spermiating male goldfish. Serum GtH concentrations are given as mean±SE. At each sampling time, serum GtH concentrations that are similar ($P>0.05$) are identified by a similar superscript.

1st Injection	2nd Injection		GtH (ng/ml) at h after 2nd injection		
(−3 h)	(0 h)	n	3	6	24
PS+vehicle	PS+vehicle	8	3.4±0.3[1]	2.9±0.3[1]	3.4±0.4[1]
PS+bromo(2)	PS+bromo(2)	8	3.4±0.6[1]	2.4±0.3[1]	2.7±0.3[1]
PS+bromo(20)	PS+bromo(20)	8	3.3±0.7[1]	3.1±0.4[1]	3.2±0.3[1]
LHRH-A+vehicle	LHRH-A+vehicle	8	32.1±6.1[2]	35.8±11.2[2]	15.7±7.4
LHRH-A+bromo(2)	LHRH-A+bromo(2)	9	26.0±6.2[2,3]	35.8±10.7[2]	19.0±7.1[2]
LHRH-A+bromo(20)	LHRH-A+bromo(20)	8	11.8±3.4[3]	14.8±4.0[2]	11.6±3.9[2]

Experiment 6.3. Effects of pimozide, phentolamine or propranolol on serum GtH concentrations.

Serum GtH concentrations were significantly higher in the pimozide than in the vehicle injected fish at 6 and 24 h after injection (Table 6.2). Serum GtH concentrations in fish injected with phentolamine or propranolol were not different from control values at all sampling times (Table 6.2).

Experiment 6.4. Effects of pimozide, phentolamine or propranolol, injected at the time of the second of two LHRH-A injections, on serum GtH concentrations.

Serum GtH concentrations of the various treatment groups at 24 h after the second LHRH–A injection are shown in Figure 6.2. Serum GtH levels in the PS + PS+vehicle, PS + PS+propranolol and PS + PS+phentolamine groups were not different from each other, but were significantly lower than those in all other treatment groups. Serum GtH levels in the PS + PS+pimozide group were significantly lower than those in the LHRH–A + LHRH–A+vehicle and LHRH–A + LHRH–A+pimozide groups, but were not different from those in the LHRH–A + LHRH–A+phentolamine and LHRH–A + LHRH–A+propranolol groups. Serum GtH values in the LHRH–A + LHRH–A+vehicle, LHRH–A + LHRH–A+phentolamine and LHRH–A + LHRH–A+propranolol groups were similar to one another, but were all significantly lower than values in the LHRH–A + LHRH–A+pimozide injected group.

Experiment 6.5. Effects of pimozide, metoclopramide or octopamine, injected at the time of the second of two LHRH-A injections, on serum GtH concentrations and ovulation.

Results of this experiment are shown in Table 6.3. Briefly, fish receiving 2 LHRH–A injections at an interval of 12 h had significantly higher serum GtH levels than PS injected fish at all sampling times. Serum GtH concentrations in the PS + PS+pimozide (10 ug/g), PS + PS+pimozide (1 ug/g) and PS + PS+octopamine (10 ug/g) groups were not significantly different from those in the PS + PS+vehicle injected fish at all sampling times. Fish injected with PS + PS+metoclopramide (10 ug/g) had serum GtH values that were significantly higher than those in the PS + PS+vehicle injected fish at 6 but not at 0, 24 and

Table 6.2. Effects of intraperitoneal injection of catecholaminergic blockers on serum GtH concentrations in goldfish undergoing gonadal recrudescence (mixed sex).

Treatment	n	GtH (ng/ml) at h postinjection		
		0[1]	6	24
vehicle	10	1.0±0.1[2]	0.9±0.1	0.8±0.1
pimozide, 10 ug/g[3]	10	1.2±0.1	1.6±0.4[4]	2.2±0.5[4]
phentolamine, 10 ug/g	10	0.9±0.1	0.8±0.1	0.6±0.1
propranolol, 10 ug/g	10	0.9±0.1	1.2±0.2	0.7±0.1

[1] Samples taken just prior to drug or vehicle injection.

[2] Mean±SE.

[3] Doses in ug/g body weight.

[4] Significantly greater than vehicle injected control, $P<0.025$.

Figure 6.2. Effects of intraperitoneal injection (ip) of pimozide (10 ug/g; PIM), phentolamine (10ug/g; PHEN) or propranolol (10 ug/g; PROP) on serum GtH concentrations in LHRH-A injected goldfish undergoing gonadal recrudescence. LHRH-A (0.1 ug/g) was injected twice at an interval of 12 h. PIM, PROP or PHEN were injected at the time of the second LHRH-A injection. Serum GtH levels were plotted as mean±SE. Serum GtH concentrations of groups underlined by the same line are similar (P>0.05).

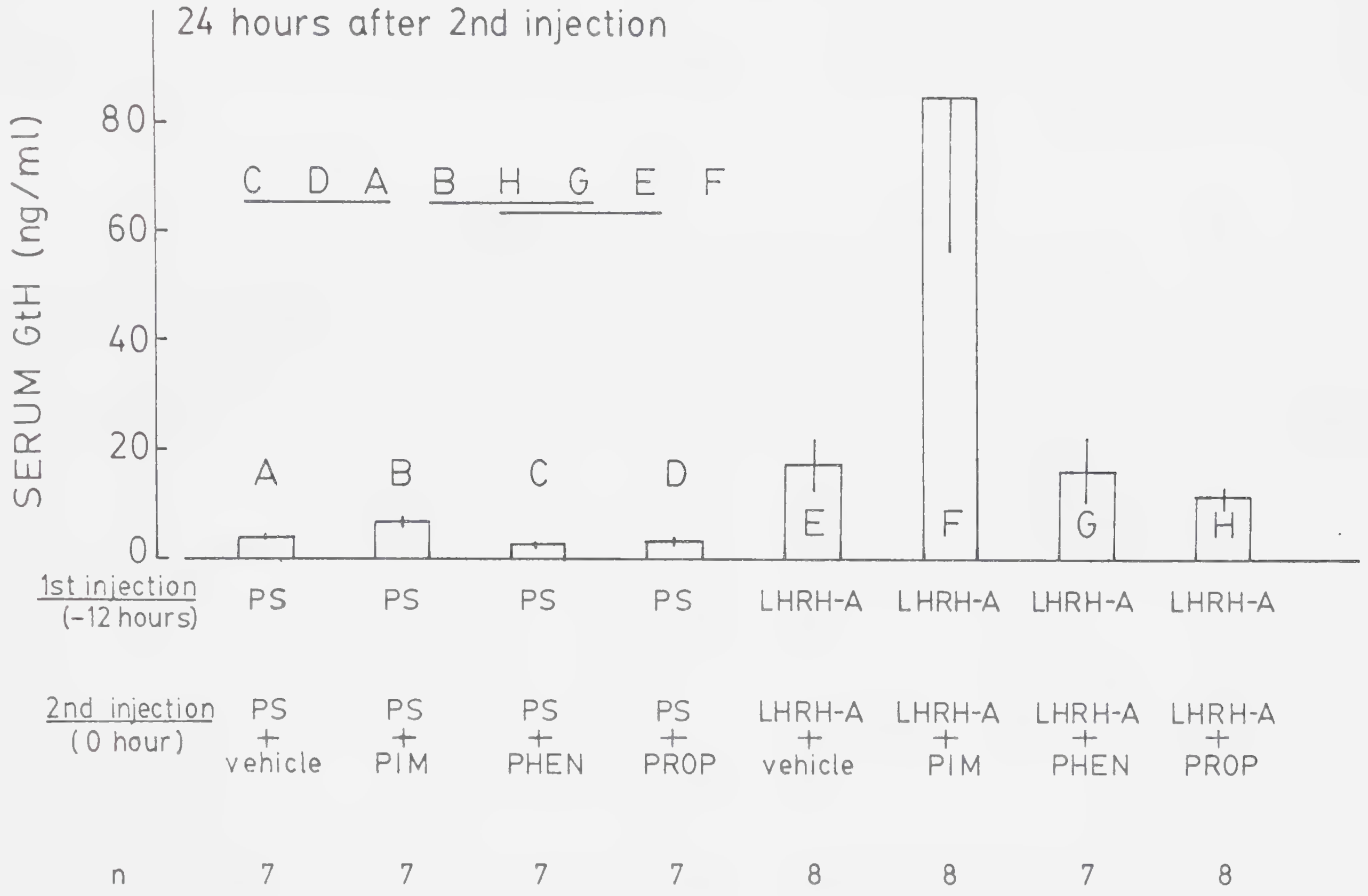


Table 6.3. Effects of intraperitoneal injection of pimozide (PIM), metoclopramide (MET) or octopamine (OCT) on serum GtH levels and ovulation in LHRH-A injected (0.1 ug/g) gravid female goldfish. PIM, MET or OCT were injected with the 2nd LHRH-A injection, 12 h after the first injection. Serum GtH concentrations are given as mean±SE. At each sampling time, serum GtH concentrations that are not significantly different (P>0.05) are identified by a similar superscript.

1st Injection	2nd Injection		GtH (ng/ml) at h after 2nd injection				Fish
(-12 h)	(0 h)	n	0	6	24	48	ovulated
PS	PS+vehicle	8	3.9±0.5[1]	3.9±0.3[1]	5.4±0.7[1,2]	4.7±0.6[1]	0
PS	PS+PIM(10)◇	7	3.3±0.5[1]	4.5±0.4[1]	15.6±7.7[1]	7.7±3.0[1]	0
PS	PS+PIM(1)	8	3.7±0.5[1]	5.1±0.8[1]	5.5±1.2[1,2]	4.2±0.5[1,2]	0
PS	PS+MET(10)	7	6.0±2.8[1]	9.2±2.2[2]	7.6±2.1[1]	5.2±1.1[1]	0
PS	PS+MET(1)	8	3.3±0.2[1]	4.2±0.5[1]	4.1±0.3[2]	3.2±0.2[2]	0
PS	PS+OCT(10)	7	3.9±0.4[1]	4.6±0.3[1]	4.5±0.4[2]	3.9±0.5[1,2]	0
LHRH-A	LHRH-A+vehicle	8	47.3±13.5[2]	73.4±21.7[3]	154±50[3]	67.4±19.6[3]	1
LHRH-A	LHRH-A+PIM(10)	7	59.5±15.9[2]	205±44[4]	342±97[4]	348±44[4]	7 ▲
LHRH-A	LHRH-A+PIM(1)	8	46.4±10.9[2]	91.5±23.1[3]	284±97[3,4]	178±54[5]	5 △
LHRH-A	LHRH-A+MET(10)	7	46.7±19.0[2]	135±41[3,4]	293±87[3,4]	250±67[4,5]	4 △
LHRH-A	LHRH-A+MET(1)	8	40.8±9.1[2]	47.3±8.3[3]	76.6±21.9[3]	39.4±15.4[3]	1
LHRH-A	LHRH-A+OCT(10)	7	32.2±8.1[2]	51.8±16.4[3]	89.8±28.9[3]	69.0±22.0[3]	0

◇ Doses in ug/g body weight.

▲ Significantly greater than all other groups.

△ Significantly greater than LHRH-A+vehicle, LHRH-A+MET(1), LHRH-A+OCT(10) and all PS injected groups.

48 h after the second injection. The LHRH–A + LHRH–A+pimozide (10 ug/g) group had significantly higher serum GtH concentrations than the LHRH–A + LHRH–A+vehicle injected group at 6, 24 and 48 h following the second injection. At 48 h following the second injection, serum GtH values in the LHRH–A + LHRH–A+pimozide (10 ug/g) group were also significantly higher than those in the LHRH–A + LHRH–A+pimozide (1 ug/g), LHRH–A + LHRH–A+metoclopramide (1 ug/g) and LHRH–A + LHRH–A+octopamine (10 ug/g), but not the LHRH–A + LHRH–A+metoclopramide (10 ug/g) group. The LHRH–A + LHRH–A+pimozide (1 ug/g) and LHRH–A + LHRH–A+metoclopramide (10 ug/g) groups had serum GtH levels that were not different from one another at any sampling time; values of both of these groups were significantly higher than those of the LHRH–A + LHRH–A+vehicle group at 48 h after the second injection. At all sampling times, serum GtH levels in the LHRH–A + LHRH–A+octopamine (10 ug/g), LHRH–A + LHRH–A+metoclopramide (1 ug/g) and LHRH–A + LHRH–A+vehicle groups were not different from each other.

Ovulation was detected only at the last sampling time. The frequencies of occurrence of ovulation in the LHRH–A + LHRH–A+vehicle and LHRH–A + LHRH–A+metoclopramide (1 ug/g) groups were not significantly different from zero. The number of ovulated fish in the LHRH–A + LHRH–A+metoclopramide (10 ug/g) and the LHRH–A + LHRH–A+pimozide (1 ug/g) groups were not different from each other, but were significantly higher than those in the LHRH–A + LHRH–A+vehicle, LHRH–A + LHRH–A+metoclopramide (1 ug/g), LHRH–A + LHRH–A+octopamine (10 ug/g) and all the PS injected groups. The LHRH–A + LHRH–A+pimozide (10 ug/g) injected group had a significantly higher number of ovulated fish than all other treatment groups.

D. Discussion

Two injections of LHRH–A, at an interval of 12 h, increased serum GtH concentrations in goldfish kept at 12°C (Figures 6.1 and 6.2, Table 6.3) as in previous experiments (Peter, 1980; Chapters III and V). Also, two injections of LHRH–A at a 3–h interval increased serum GtH concentrations in goldfish kept at 12°C, similar to previous investigations using goldfish kept at 20°C (Sokolowska *et al.*, 1983). Two injections of a low dosage of LHRH–A stimulated greater GtH release than a single injection of a large

dose (Peter, 1980), indicating that the GtH-release response in goldfish can be potentiated by a previous priming injection of the releasing hormone.

Results from Chapter V showed that in goldfish injection of pimozide with the first or the second of two LHRH-A injections potentiated the ability of the peptide to increase GtH release and to induce ovulation. In addition, it was shown that simultaneous injection of the DA agonist apomorphine with the two injections of LHRH-A reduced the GtH release-response to the peptide (Chapter III). In the present work, injection of the DA agonist apomorphine either at the time of the first or the second of two LHRH-A injections was found to significantly depress the LHRH-A stimulated increase in serum GtH (Figure 6.1). The increased GtH release caused by two LHRH-A injections was reduced by simultaneous administration of bromocriptine (20 ug/g; Table 6.1) with both LHRH-A injections. Bromocriptine is a specific D-2 DA agonist in mammals (Kebabian and Calne, 1979) and has been shown to inhibit LH release in rats (Fuxe *et al.*, 1978). Together, these observations indicate that stimulation of DA receptors can block the potentiating effects of multiple doses of the releasing hormone on GtH release in goldfish, as well as block ongoing LHRH-A stimulated release. The results also suggest that the DA inhibition of GnRH actions and GtH release may be mediated by receptors similar to the mammalian D-2 receptors.

The influences of various catecholaminergic agonists and antagonists on GtH release in normal and LHRH-A injected goldfish were monitored to determine if the DA inhibition of GtH release can be explained by non-specific stimulation of alpha- or beta-adrenegic receptors. Metoclopramide is a specific antagonist for the mammalian D-2 DA receptor (Kebabian and Calne, 1979) and has been shown to increase circulating LH levels in women (Quigley *et al.*, 1979). In this study, intraperitoneal injection of a 10 ug/g dose of metoclopramide elevated the basal serum GtH levels, and potentiated the GtH release-response to LHRH-A in goldfish (Table 6.3). Intraperitoneal injections of pimozide alone at 10 ug/g raised basal serum GtH concentrations, and the administration of pimozide at both 1 and 10 ug/g increased the GtH release-response to injections of LHRH-A (Figure 6.2, Tables 6.2 and 6.3), similar to results from previous experiments (Chapters III and V). However, in both normal and LHRH-A injected fish, injection of phentolamine (alpha-antagonist), propranolol (beta-antagonist) and octopamine (alpha-adrenergic

sympathomimetic agent that is also found endogeneously in goldfish; Axelrod and Saavedra, 1977) did not alter serum GtH levels (Figure 6.2, Tables 6.2 and 6.3). In goldfish, intraperitoneal injection of the alpha-adrenergic agonist, clonidine, increases serum GtH levels (Chapter II) and NE stimulates GtH release via alpha-receptors (see Chapter VII for NE results). Together, these observations indicate that the GtH release-inhibitory activity of DA is specific and cannot be explained by nonspecific actions on alpha- or beta-adrenergic receptors, or by conversion of DA to NE. The results with intraperitoneal injections of the high dose of pimozide and metoclopramide also support the idea that DA inhibition of GtH secretion in goldfish is mediated by a D-2 - like receptor.

In two experiments of the present study, intraperitoneal injection of pimozide at 10 ug/g significantly increased serum GtH levels at 24 h postinjection compared to vehicle injected control goldfish (Figure 6.1, Table 6.2). However, in another experiment the serum GtH levels at 24 h following a similar dose of pimozide were not significantly greater than control values (Table 6.3), although the levels were significantly higher than values measured at the time of pimozide injection ($P<0.01$, 2-tailed paired-t test). These results indicate that intraperitoneal injection of pimozide at 10 ug/g consistently increases serum GtH concentrations at 24 h postinjection as in previous studies (Chapters III and IV). A significant increase in serum GtH levels in response to intraperitoneal injections of a similar dose (10 ug/g) of metoclopramide was only observed at 6 h, but not 24 h, postinjection (Table 6.3). Since pimozide was injected as a suspension while metoclopramide was administered as a solution, the rate of uptake and metabolism of the two DA antagonists would be different; therefore, the GtH response to pimozide injection would likely have a slower onset but longer duration than the response to the injection of a similar dose of metoclopramide.

The frequency of ovulation as well as serum GtH concentrations in the LHRH-A + LHRH-A+pimozide (10 ug/g) group was significantly higher than those in the LHRH-A + LHRH-A+pimozide (1 ug/g) and LHRH-A + LHRH-A+metoclopramide (10 ug/g) groups (Table 6.3). Intraperitoneal injections of metoclopramide at 10 ug/g or pimozide at 1 ug/g were equipotent in potentiating LHRH-A induced GtH release and ovulation in gravid female goldfish (Table 6.3). These differences may be explained by the probable differential rates of absorption and metabolism referred to above, as well as possible differences in the

relative potency of the two DA antagonists in blocking DA actions. Pimozide is 25-times more potent than metoclopramide in reversing the DA inhibition of cAMP levels in the rat pituitary gland (Meunier and Labrie, 1982). On this basis it is concluded that pimozide, being longer lasting (due to its mode of administration as a suspension) and more potent than metoclopramide, is more effective for a longer time in removing the DA inhibition of GtH secretion.

In this study, the LHRH-A induced elevation in serum GtH concentrations was significantly reduced by bromocriptine (20 ug/g) at 3 h but not at 6 or 24 h, following the second of two injections of the DA agonist. On the other hand, the GtH release response to LHRH-A injections was significantly depressed by apomorphine (20 ug/g) at 18 and 24 h, but not at 6 or 12 h, after injection (Figure 6.1, Table 6.1). These results indicate that bromocriptine may be a faster acting DA agonist than apomorphine in goldfish.

In a previous study (Chapter III), intraperitoneal injection of apomorphine was effective in decreasing serum GtH concentrations in normal, as well as in LHRH-A injected goldfish. However, in the current report, apomorphine and bromocriptine only significantly decreased serum GtH levels in LHRH-A treated goldfish (Figure 6.1, Table 6.1). In Chapter III, it was suggested that in normal goldfish the endogenous inhibitory influence on GtH release might be at or near maximum, and the capacity of the gonadotrops to further decrease GtH secretion in response to increased stimulation of DA receptors might be limited. This may explain the failure of apomorphine and bromocriptine injections to depress circulating GtH levels in intact goldfish in the present investigation.

At 48 h after metoclopramide or vehicle injection, serum GtH concentrations of the PS + PS+metoclopramide (1 ug/g) injected group were significantly lower than those of the PS + PS+vehicle injected group (Table 6.3); however, serum GtH levels of both treatment groups were not significantly different from their own respective values at the time of injection. In view of the fact that serum GtH concentrations in the PS + PS+metoclopramide (1 ug/g) group were somewhat lower than those in the PS + PS+vehicle group at the time of metoclopramide injection, the apparent anomaly with the injection of the low dose of metoclopramide does not provide evidence against the working hypothesis that DA has GtH release-inhibitory activity.

In summary, results from this study are consistent with the idea that DA has GtH release–inhibitory activity in goldfish, in part, by blocking the actions of GnRH. Observations from this study indicate that the DA inhibition of GtH release is specific and suggest that a receptor similar to the mammalian D–2 type may mediate this inhibitory influence. Although metoclopramide is less effective than pimozide in potentiating LHRH–A induced GtH release and ovulation in the goldfish, both DA antagonists are potentially useful tools for use with LHRH–A to induce ovulation in cultured fishes.

VII. Influences of Norepinephrine and Alpha-adrenergic Mechanisms on Gonadotropin Secretion in Female Goldfish, *Carassius auratus*.

A. Introduction

As reviewed in the General Introduction (Chapter I), studies on mammalian species have consistently shown that NE stimulates LH release by increasing LHRH secretion from the median eminence; the stimulatory influence of NE on LH and LHRH release is mediated by alpha-adrenergic receptors.

In teleosts, few data are available on the possible influences that NE may have on GtH release. Deery (1975) reported that epinephrine and NE stimulated the *in vitro* adenyl cyclase activity of the pars distalis of goldfish. Intraperitoneal injection of clonidine, an alpha-adrenergic agonist, increased serum GtH levels in goldfish at early stages of ovarian recrudescence (Chapter II). It is possible that NE may increase GtH release via alpha-receptors as in mammals. In the present study, the possible involvement of NE and alpha-adrenergic mechanisms on the neuroendocrine regulation of GtH release in female goldfish were further investigated by monitoring serum GtH concentrations following injection of clonidine, phentolamine (alpha-blocker), and NE.

B. Materials and Methods

Female goldfish were held on a similar photoperiod-temperature regime as described in Chapter II. Fish maintenance, handling and blood sampling procedures outlined in Chapter II were followed. Serum GtH concentrations were measured as described in Chapter IV.

Norepinephrine HCl (NE; Sigma), clonidine and phentolamine were made up fresh in a vehicle of acidified 0.7% saline with 0.1% sodium metabisulfite, kept on ice, and injected either intraperitoneally or intraventricularly according to procedures described in Chapter III. Control groups were given an equivalent volume of the vehicle. Brains of fish receiving intraventricular injection were fixed in Bouin's at the end of the experiment and processed for histological examination according to procedures given in Chapter III. Brain sections through the preoptic region were examined but no damage was evident due to the injection procedure.

Paired t test was used to compare pretreatment and posttreatment serum GtH levels within experimental groups. The Mann–Whitney U test was used to compare GtH concentrations between groups (Snedecor and Cochran, 1971).

Details of experimental treatments are summarized in Table 7.1.

C. Results

Experiment 7.1. Effects of intraperitoneal injection of various doses of NE on serum GtH concentrations in female goldfish.

The effects of intraperitoneal injection of NE on serum GtH levels were examined in several experiments using female goldfish at several stages of ovarian recrudescence. Because the GtH concentration changes throughout the ovarian recrudescence cycle of the goldfish, the serum GtH levels at 0.5 h after vehicle or NE injection were expressed as a % of the preinjection values to facilitate comparisons of the NE effects on circulating GtH levels between replicate experiments (Figure 7.1). In goldfish with regressed ovaries (July experiment), intraperitoneal injection of NE at 1, 10 or 100 ug/g significantly increased serum GtH levels at 0.5 h postinjection compared to pretreatment values. In comparison with the vehicle injected control (0 ug/g group), injection of NE at 1 ug/g in fish with regressed ovaries also increased serum GtH levels at 0.5 h postinjection. In goldfish in early ovarian recrudescence (November experiment), intraperitoneal injection of NE at 10 or 100 ug/g significantly raised serum GtH concentrations at 0.5 h postinjection compared to both control (0 ug/g group) and preinjection values. In goldfish that were at mid– or late ovarian recrudescence (March and May experiments) and in goldfish with ovaries undergoing regression (June experiment), intraperitoneal injection of various doses of NE did not alter serum GtH levels at 0.5 h postinjection compared to control or preinjection values.

Experiment 7.2. Effects of intraventricular injection of NE on serum GtH concentrations in female goldfish.

In comparison with pretreatment and control values, intraventricular injection of NE at 20 ug/fish did not alter serum GtH concentrations at 2 h postinjection in fish at early

Table 7.1. Summary of experimental groups.

Experiment	Drugs tested	Time of Year	GSI (%)	Sample times (h postinjection)
7.1	NE; doses vary between 0.1, 1, 10, 100 ug/g,[3] ip[4]	May	9.8±0.7[1]	0[2], 0.5
		June	5.7±0.5	0, 0.5
		July	1.6±0.3	0, 0.5
		November	2.7±0.2	0, 0.5
		March	7.8±0.6	0, 0.5
7.2	NE; 2, 20 ug/fish, iv[4]	November	2.6±0.2	0, 2
		February	6.2±0.7	0, 2
7.3	phentolamine, 10 ug/g, ip and/or NE, 100 ug/g.ip	January	4.0±0.4	0.5
7.4	clonidine; 30 ug/g, ip	April	10.8±0.9	0, 6, 24

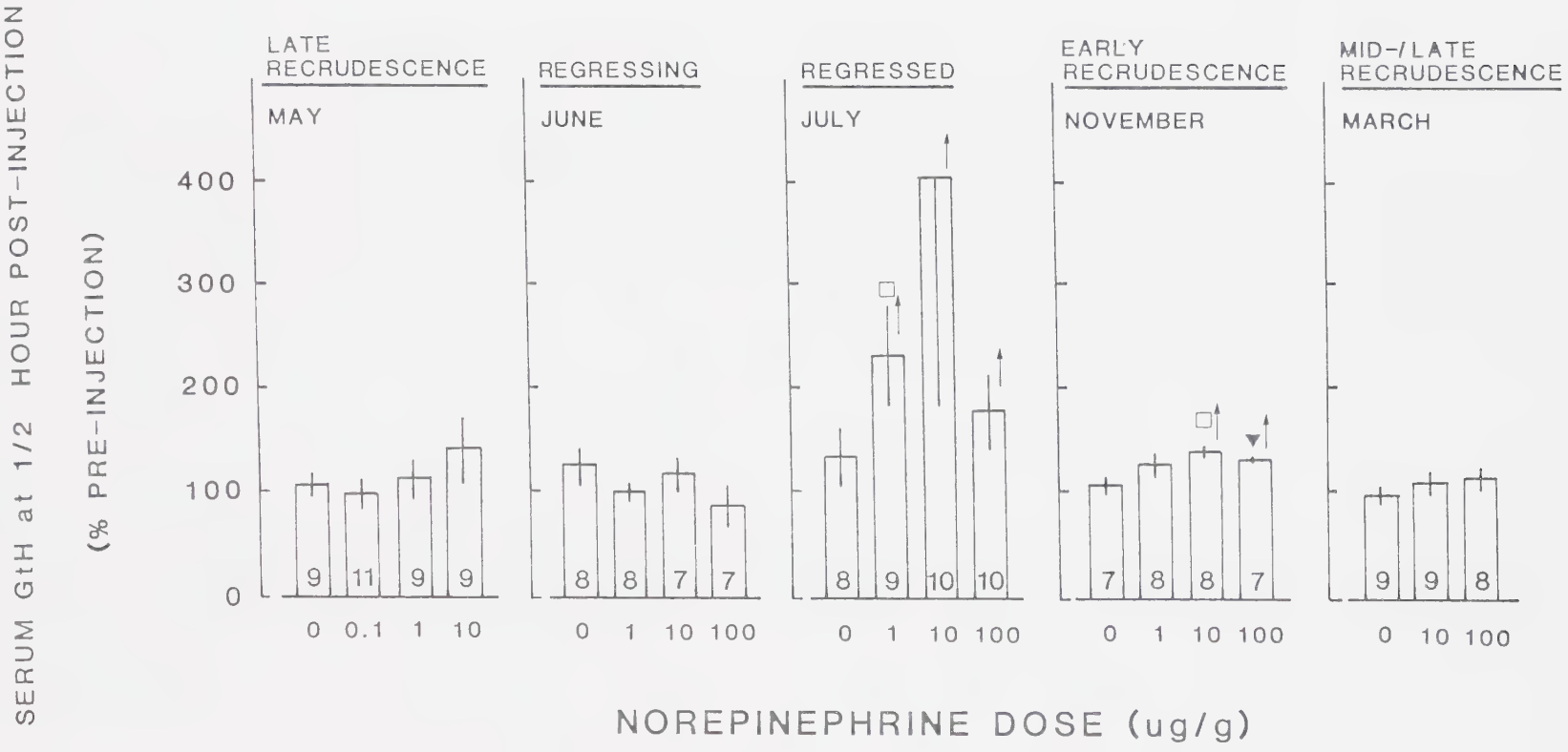
[1] Mean±SE.

[2] Samples taken just prior to drug or vehicle injection.

[3] Doses as ug/g body weight.

[4] ip, intraperitoneal injection; iv, intraventricular (3rd cranial ventricle) injection.

Figure 7.1. Effects of intraperitoneal injection of various doses of NE on serum GtH concentrations in female goldfish at several stages of ovarian recrudescence. For each treatment group, serum GtH levels at 0.5 h postinjection were expressed as % of their own preinjection values. Serum GtH levels are plotted as mean±SE. Dosages are given as ug/g body weight. Vehicle injected controls are shown as 0 ug/g NE. (Average preinjection GtH values, mean±SE, for the May, June, July, November, and March experiments were 3.7±0.3, 5.0±0.4, 1.0±0.1, 3.8±0.2, and 2.8±0.2 ng/ml, respectively.) ↑, $P<0.05$ vs preinjection; □, $P<0.05$ vs control, 0 ug/g; ▲, $P<0.01$ vs control, 0 ug/g.



(November experiment) or mid– ovarian recrudescence (February experiment) (Table 7.2). Intraventricular injection of NE at 2 ug/fish significantly increased serum GtH levels at 2 h postinjection in female goldfish in early but not in mid– ovarian recrudescence compared to vehicle injected controls; however, compared to pretreatment values, intraventricular injection of NE at 2 ug/fish did not alter serum GtH concentrations in fish at early or mid–ovarian recrudescence (Table 7.2).

Experiment 7.3. Effects of intraperitoneal injection of NE and/or phentolamine on serum GtH concentrations in female goldfish.

Fish used in this experiment ranged from early to mid– ovarian recrudescence. Intraperitoneal injection of NE at 100 ug/g significantly increased serum GtH concentration at 0.5 h postinjection compared to vehicle injected controls (Figure 7.2). In comparison with control values, intraperitoneal injection of phentolamine (10 ug/g) did not alter serum GtH concentrations at 0.5 h postinjection. At 0.5 h postinjection, serum GtH concentrations in fish injected simultaneously with NE (100 ug/g) and phentolamine (10 ug/g) were significantly lower than values in fish injected with either NE (100 ug/g) or phentolamine (10 ug/g; Figure 7.2).

Experiment 7.4. Effects of intraperitoneal injection of clonidine on serum GtH concentrations in female goldfish.

Intraperitoneal injection of clonidine did not alter serum GtH concentrations at 6 and 24 h postinjection compared to control or preinjection values (Table 7.3). In the same experiment, serum GtH concentrations in the vehicle injected controls were significantly higher than pretreatment values at 6 and 24 h postinjection.

D. Discussion

In the present study, intraperitoneal injection of NE increased serum GtH concentrations in female goldfish with regressed ovaries or in fish with ovaries at early stages of ovarian recrudescence (July and November experiments, Figure 7.1; January experiment, Figure 7.2). Intraperitoneal injection of NE had no effect on serum GtH levels when tested in female goldfish at other times of the ovarian recrudescence/regression

Table 7.2. Effects of intraventricular injection of NE on serum GtH concentrations in female goldfish.

Gonadal	Treatment		GtH (ng/ml) at h postinjection	
Condition		n	0	2
A)				
Early recrudescence	vehicle	11	2.3±0.4[1]	1.9±0.3
(November)	NE 2 ug/fish	10	2.9±0.6	4.4±1.2[2]
	NE 20ug/fish	11	3.5±0.8	2.1±0.4
B)				
Mid- recrudescence	vehicle	9	9.8±1.2	8.0±1.2
(February)	NE 2 ug/fish	7	11.1±1.6	7.8±1.5
	NE 20ug/fish	7	11.6±1.5	11.3±2.3

[1] Mean±SE.

[2] Significantly greater than control, $P<0.025$.

Figure 7.2. Effects of intraperitoneal injection of NE (100 ug/g body weight) and/or phentolamine (PHEN, 10 ug/g body weight) on serum GtH concentrations in female goldfish. Serum GtH values are plotted as mean±SE. △, $P<0.05$; ⊠, $P<0.01$; ▲, $P<0.005$.

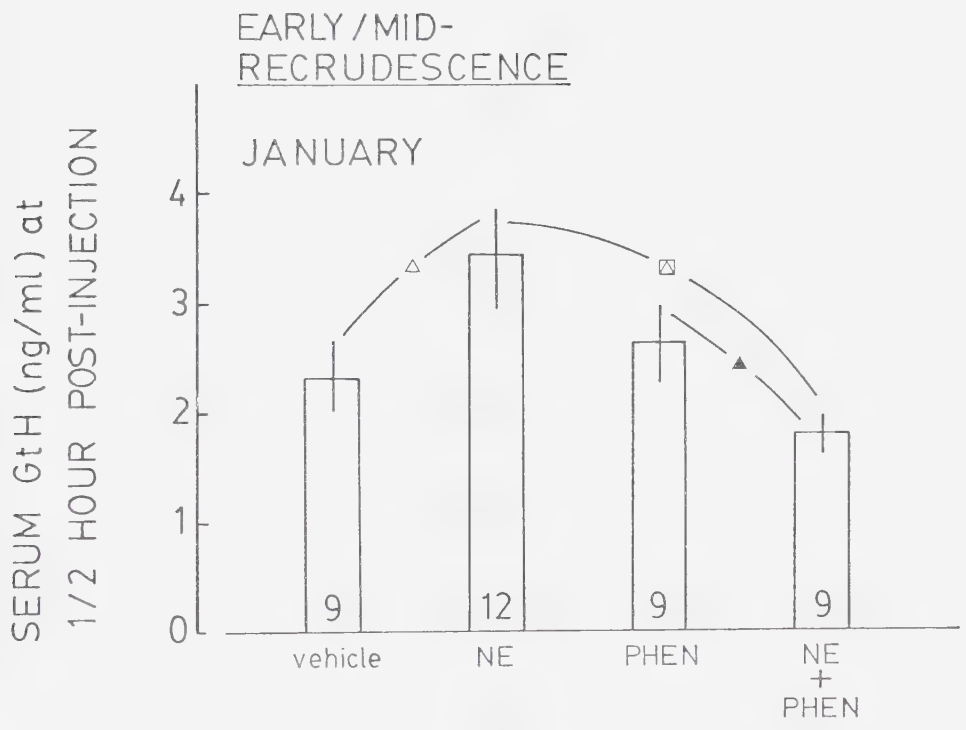


Table 7.3. Effects of intraperitoneal injection of clonidine (30 ug/g) on serum GtH concentrations in female goldfish.

Gonadal Condition	Treatment	n	GtH (ng/ml) at h postinjection		
			0	6	24
Late recrudescence (April)	vehicle	7	3.0±0.5[1]	4.0±1.0	6.0±1.5[2]
	clonidine	6	6.4±2.5	5.4±1.8	7.3±2.6

[1] Mean±SE.

[2] Significantly greater than pretreatment values, $P<0.025$.

cycle (March, May and June experiments, Figure 7.1). In mammals, the existence of a blood–brain barrier for NE is well documented (Glowinski *et al.*, 1964). A blood–brain barrier for NE probably also exists in the goldfish as systemically injected radio–labelled NE was not taken up readily by brain tissues (J. Chang and R. Peter, unpublished results). Since the pituitary lies outside of the blood–brain barrier, these results with intraperitoneal injection of NE suggest that in goldfish NE may act on the pituitary to increase GtH release at times of the year when the ovaries are regressed or in early stages of recrudescence.

The reason for this seasonality of the GtH response to NE is not known, but may be due to seasonal differences in steroid influences on GtH release. Changes in circulating levels of sex steroids with the gonadal recrudescence/regression cycle have been found in goldfish (Schreck and Hopwood, 1974). Mammalian LHRH and its analogues have been shown to have decreased ability to stimulate GtH release in fish with regressed ovaries or ovaries at early stages of recrudescence (for review see Peter, 1982b, 1983). How the changing responsiveness to NE and GnRH may relate to changes in steroid hormone levels is not known at this time.

In mammals, NE stimulates LH release by action on central alpha–receptors to increase LHRH secretion and not by direct action on the pituitary (Ryu *et al.*, 1980; Barraclough and Wise, 1982). Intraventricular injection of NE has been shown to increase LH release in rats (Kreig and Sawyer, 1976; Vijayan and McCann, 1978). Intraventricular injection of a low dose of NE (2 ug/fish) increased serum GtH levels in female goldfish at early ovarian recrudescence (Table 7.2, A). This suggests that NE may also act centrally, perhaps by stimulating GnRH secretion, to increase GtH release in the goldfish. Intraventricular injection of NE did not alter serum GtH concentrations in female goldfish at mid– ovarian recrudescence (Table 7.2, B), which may be a reflection of a seasonal variation in the action of NE on central sites, similar to the results with intraperitoneal injections. The reason for the failure of the higher dose of NE (20 ug/fish) to illicite a GtH response in goldfish at early ovarian recrudescence (Table 7.2, A) is not known. However, this may be the result of stimulation, by the higher dose of NE, of more than one target site or effector system, culminating in the cancellation of the stimulatory influence of NE on GtH release.

Injection of clonidine, an alpha-agonist that crosses the blood-brain barrier, increases LH release in mammals (Estes *et al.*, 1982). In Chapter II, it was shown that intraperitoneal injection of clonidine (30 ug/g) increased serum GtH concentrations in female goldfish with gonads at early (October) or mid- ovarian recrudescence (February). However, in the present study the same dose of clonidine did not alter serum GtH concentrations in goldfish at late ovarian recrudescence (Table 7.3). This seasonal variation in the serum GtH response to injections of clonidine parallels the changes in the ability of intraperitoneally or intraventricularly injected NE to increase serum GtH concentration in female goldfish at different stages of the ovarian recrudescence/regression cycle. These results suggest that the seasonal stimulatory influence of NE on GtH release may be mediated by alpha-receptors.

In ovariectomized rats, injection of the alpha-adrenergic receptor blocker, phentolamine, inhibited pulsatile LH release (Weick, 1978). In female goldfish with ovaries at early or mid- recrudescence, phentolamine did not alter circulating GtH levels when injected alone, but blocked the NE-induced increase in serum GtH concentrations when administered simultaneously with the NE injection (Figure 7.2). This finding is consistent with the hypothesis that NE stimulates GtH release in goldfish via alpha-adrenergic receptors.

In summary, results from the present study suggest that NE may stimulate GtH release in goldfish by direct actions on the pituitary as well as by central actions. The stimulatory influence of NE on GtH release is seasonal, dependent on the time of year and/or the state of ovarian recrudescence, and is mediated by alpha-adrenergic mechanisms. *In vitro* experiments (Chapter VIII) were done to investigate the direct action of NE on GtH release in goldfish.

VIII. Influences of Dopamine, Norepinephrine and LHRH-A on GtH Release *in vitro*.

A. Introduction

As outlined in the General Introduction (Chapter I), the majority of the data from studies on mammals suggests that neurotransmitters alter LH release by changing the rate of LHRH secretion. However, results from *in vivo* experiments described in Chapters III and IV indicate that in goldfish, DA inhibits the spontaneous release of GtH and directly interferes with the actions of GnRH on gonadotrops. The observation that intraperitoneal injections of NE increases serum GtH concentration (Chapter VII) suggests that NE may also have direct influences on GtH secretion from gonadotrops in goldfish.

D. MacKenzie (personal communication) has demonstrated that goldfish pituitary fragments and pituitary cells dispersed by controlled enzyme digestion continue to release GtH spontaneously *in vitro*. Perifusion of dispersed pituitary cells or pituitary fragments with neurotransmitters may be a useful *in vitro* model for studying the direct actions of these factors on GtH release. In this chapter, the influences of DA on basal GtH release from goldfish gonadotrops were studied by measuring the GtH content in perifusate of dispersed pituitary cells and pituitary fragments during and following perifusion with DA. The effects of DA on GnRH actions were investigated by monitoring the changes in GtH content of perifusate of dispersed pituitary cells and pituitary fragments during and following exposure to LHRH-A alone, LHRH-A and DA, or LHRH-A and metoclopramide (DA-antagonist). The influences of NE on basal GtH secretion were studied by monitoring alterations in GtH content of perifusate of dispersed pituitary cells during and following exposure to NE.

B. Materials and Methods

Photoperiod-temperature regime and procedures for fish maintanence and handling were as described in Chapter II. Solutions required for the *in vitro* experiments were prepared as described in Table 8.1.

Table 8.1. Summary of solutions used in *in vitro* studies.

	Hepes–Pucks	Enzyme	Complete Medium	Hepes Hanks[1]
EDTA (g)		0.07		
NaCl (g)	8.00	8.00		8.00
KCl (g)	0.40	0.40		0.40
$CaCl_2$ (g)				0.14
$Na_2HPO_4.7H_2O$ (g)	0.09	0.09		0.11
$MgSO_4.7H_2O$ (g)				0.20
KH_2PO_4 (g)	0.06	0.06		0.06
glucose (g)	1.00	1.00		1.00
Hepes, free acid (g)[2]	3.57	3.57	3.57	3.57
bovin serum albumin (g)[2]		4.00		
chicken serum (ml)[3]		10.00		
calf serum (ml)[3]			75.00	
fetal calf serum (ml)[3]			15.00	
nystatin (g)[2]			0.01	
Trypsin, EC.3.4.21.4 (g)[2]		1.00		
DNase II, EC.3.1.4.6 (g)[2]		0.03		
distilled H_2O (litre)	1.00	1.00		1.00
medium–199 (litre)[3],			1.00	
pH	7.20	7.20	7.20	7.20

1 Supplemented with 0.1% bovine serum albumin for experiments with pituitary fragments.

2 Purchased from Sigma.

3 Purchased from Gibco Canada, Calgary, Alberta.

Dispersed Cells

Pituitaries were removed from 30 female goldfish undergoing or having completed ovarian regression (July; GSI of a few representative fish ranged from <2% to 14%) and the pituitary cells dispersed following procedures outlined in Figure 8.1. Approximately three million cells were harvested. The viability following dispersion was greater than 90% as determined by the trypan blue exclusion test. The cell suspension was divided evenly among 2 polypropylene Econo-columns (Bio-Rad Lab. (Canada) Ltd., Mississauga, Ontario; id=0.8 cm; length=4 cm), each containing a layer of Biocarrier beads (Bio-Rad; bed volume=400 ul) that had been swollen in medium with serum (complete medium). The cells were allowed to settle and a further 400 ul of swelled Biocarrier beads layered on top of the cells. The cells were perifused with complete medium in a closed system (Figure 8.2, A; flow rate=20 ml/h) and allowed to recover for 3 days.

Prior to drug testing, a reservoir (50 ml syringe barrel), filled with Hepes-Hanks solution, was connected to the top of each column (Figure 8.2, B) and the complete medium replaced with Hepes-Hanks solution. The cells were perifused for 2 h with Hepes-Hanks solution, with the aid of a peristaltic pump, prior to drug testing. This allowed a 'basal' secretion rate to be established (D. MacKenzie, personal communication). LHRH-A, DA, and NE were dissolved in Hepes-Hanks solution just before use. Solutions to be tested were placed into the reservoir and the cells perifused with a series of solutions (flow rate=20 ml/h) as outlined in Table 8.2. The perifusate was collected as 10 minute (3.3ml) fractions. The fractions were stored at -28°C and their GtH content determined by radioimmunoassay as described in Chapter IV.

Pituitary Fragments

Hepes-Hanks solution, supplemented with 0.1% bovine serum albumin (albumin supplemented Hepes-Hanks) was used for these experiments. Pituitaries were removed from goldfish and pituitary fragments prepared and transferred into perifusion columns as outlined in Figure 8.3. Perifusion columns for these experiments were 3-ml dispsosable syringe barrels, each containing a layer of Biocarrier beads (prepared as in previous section) on top of a piece of filter paper (Figure 8.4). The pituitary fragments were allowed to settle on top of the Biocarrier beads and the reserviors connected to the top

Figure 8.1. Summary of cell dispersion procedure.

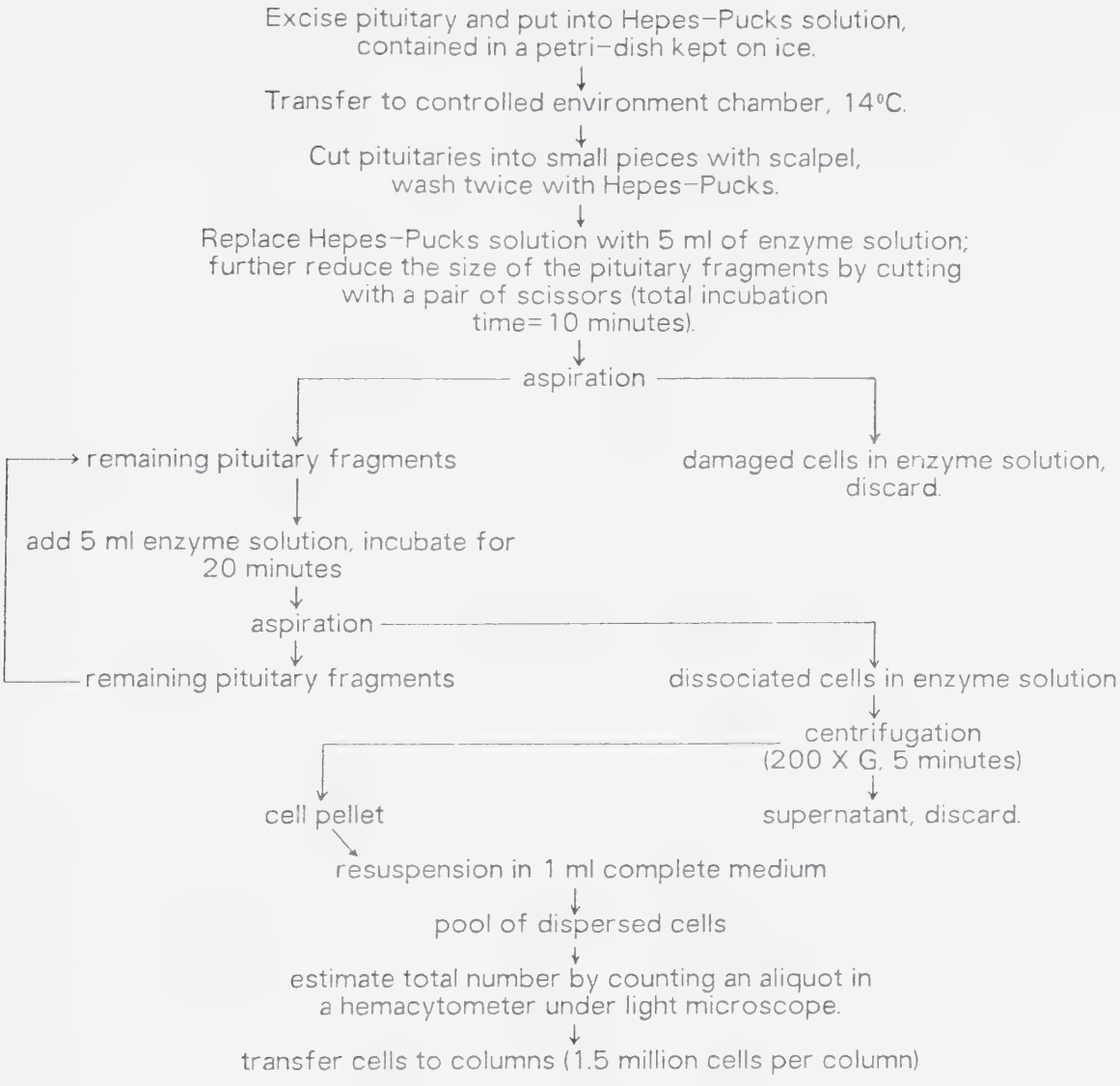


Figure 8.2. Schematic representation of the perifusion system for dispersed pituitary cells.

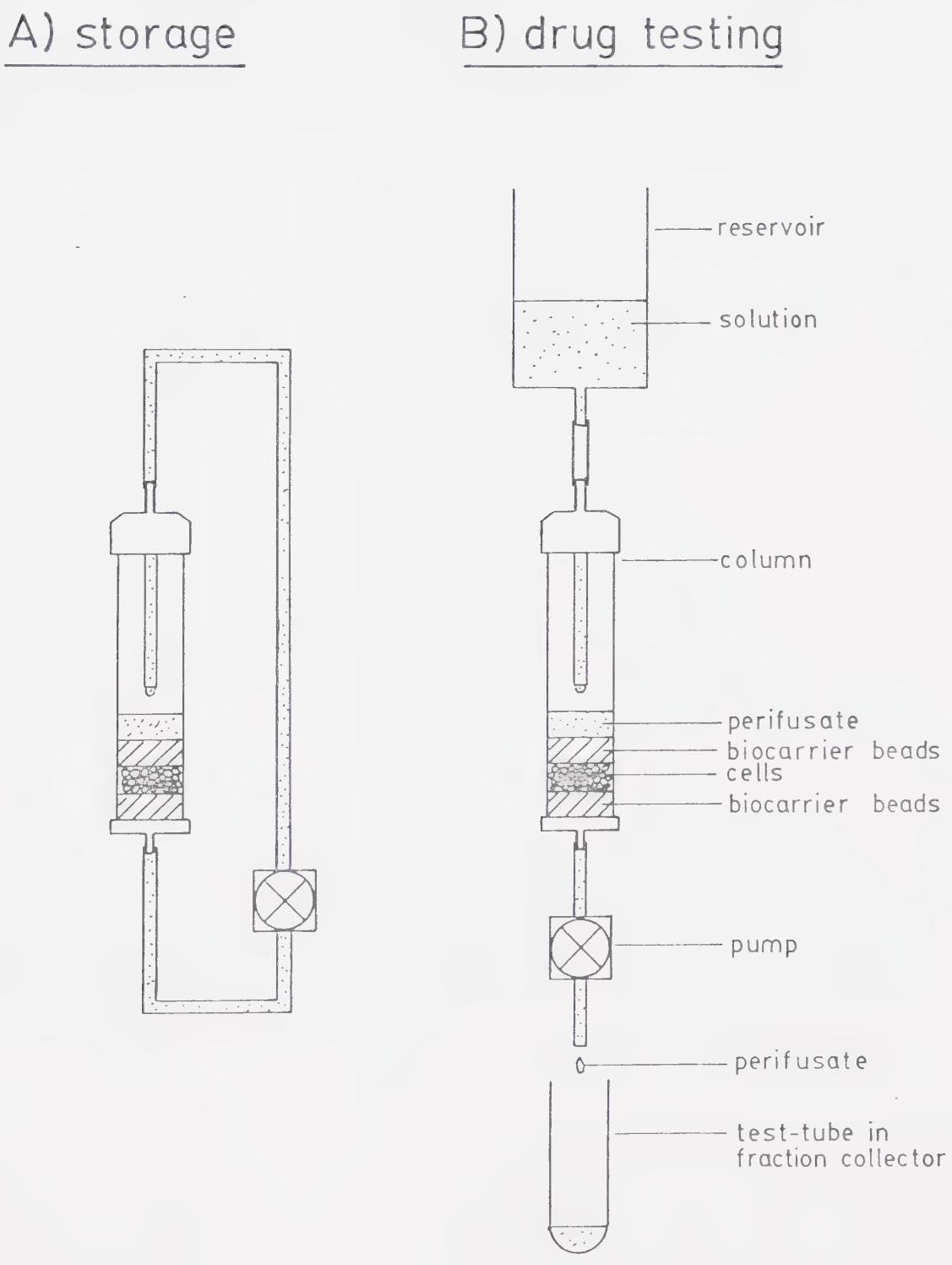


Table 8.2. Summary of experimental treatments of dispersed pituitary cells.[1]

Column	run	Solutions tested	Fractions collected
I	A	Hepes–Hanks	1
		NE (5 nM)	3
		NE (50 nM)	3
		NE (500 nM)	3
		Hepes–Hanks	1
	B	Hepes–Hanks	1
		LHRH–A (10 nM)+DA (500 nM)	3
		LHRH–A (10 nM)	2
II	A	Hepes–Hanks	1
		DA (5 nM)	3
	B	Hepes–Hanks	1
		DA (500 nM)	3
		Hepes–Hanks	1
	C	Hepes–Hanks	1
		LHRH–A (10 nM)+DA (500 nM)	3
		LHRH–A (10 nM)	1

[1] All experimental runs were completed in the same day. Cells were perifused with Hepes–Hanks solution between runs.

Figure 8.3. Summary of preparation of pituitary fragments for perifusion.

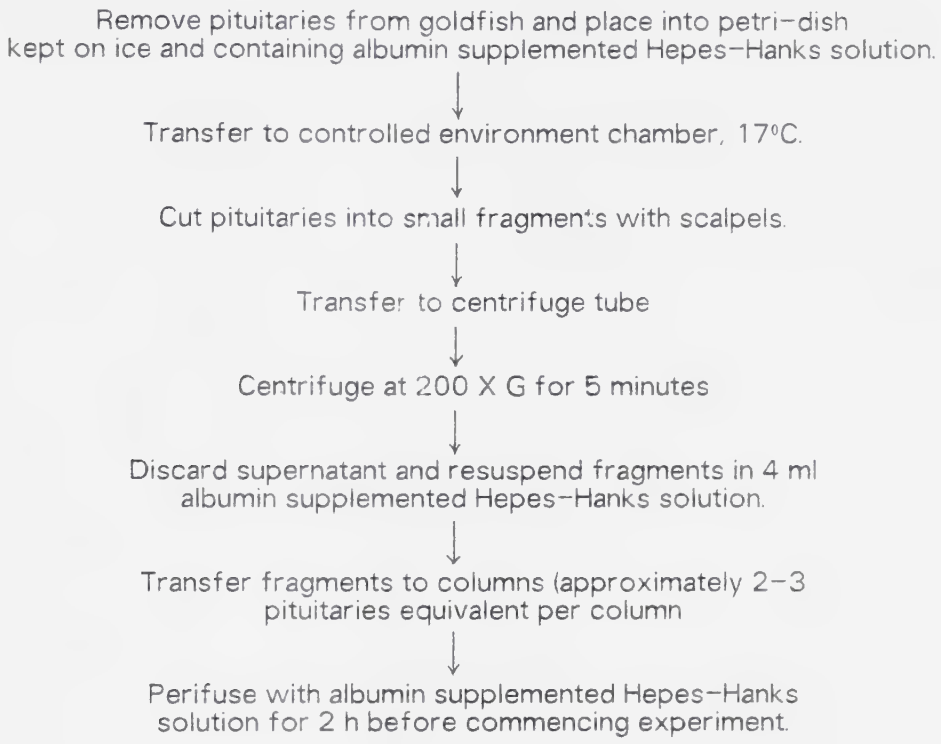
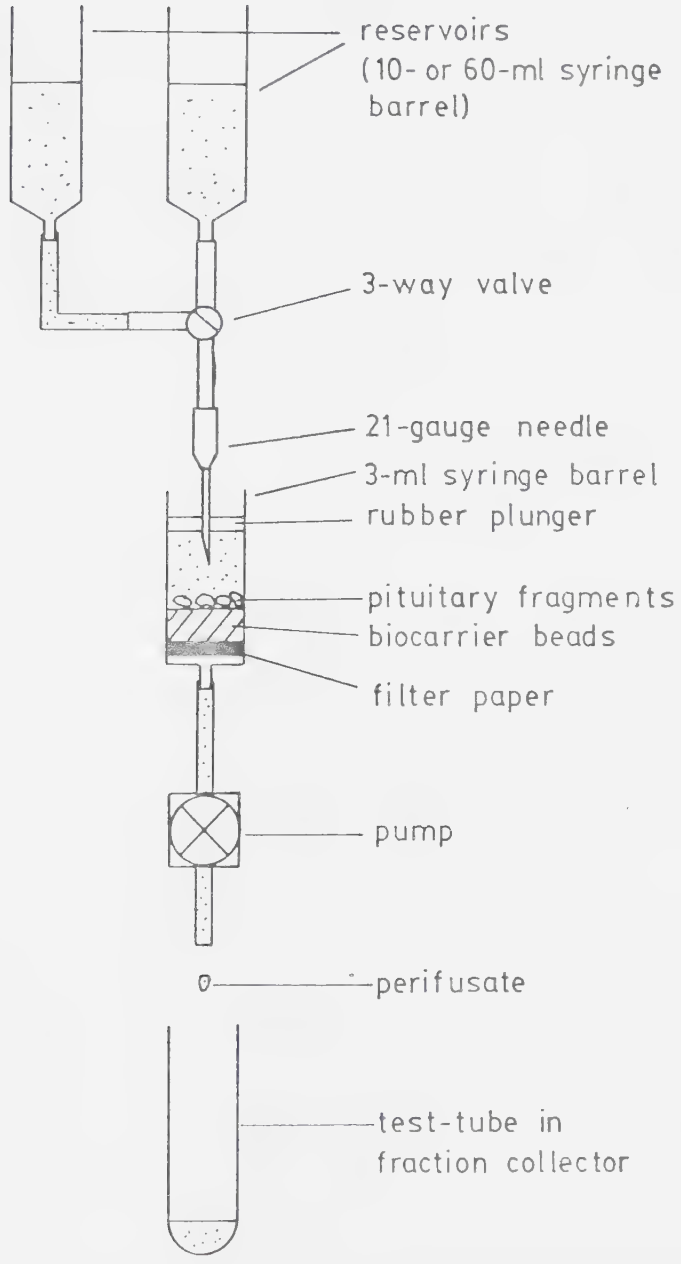


Figure 8.4. Schematic representation of the perifusion system for pituitary fragments.



of the columns as shown in Figure 8.4. The pituitary fragments were perifused with albumin supplemented Hepes–Hanks solution (flow rate= 15 ml/h) prior to drug testing. This allowed a 'basal' secretion rate to be established (D. MacKenzie, personal communication). LHRH–A, DA, and metoclopramide were dissolved in albumin supplemented Hepes–Hanks solution just before use. Solutions to be tested were placed into one of the two reserviors and the pituitary fragments perifused with a series of solutions as outlined in Table 8.3. The switching over of one perifusion solution to the next solution in the series was done by means of a 3–way valve. The perifusate was collected in 5 or 10 minute fractions (see Table 8.3 for details). The fractions were stored at −28°C and their GtH content determined by radioimmunoassay as described in Chapter IV.

C. Results

Dispersed Cells

There was no appreciable difference in the amount of GtH contained in fractions collected from column I during perifusion with Hepes–Hanks and 5 nM NE solutions (Figure 8.5). Fractions collected between 10 and 20 minutes after commencing perifusion with 50 or 500 nM NE solutions had higher GtH contents (>660 ng/fraction) than those (28 to 35 ng/fraction) in fractions collected at any other time during the same perifusion run (Figure 8.5).

Fractions collected during perifusion with DA at 5 and 500 nM had lower GtH contents than those obtained prior to DA administration (Figure 8.6). The amount of GtH contained in the fraction obtained during the last 10 minutes of perifusion with 500 nM DA was not detectable (<0.9 ng/fraction), whereas the fraction collected immediately following the termination of perifusion with DA had detectable quantities of GtH (18 ng/fraction; Figure 8.6).

The GtH contents in fractions perifused with Hepes–Hanks solution were higher than in following fractions perifused with a solution containing both LHRH–A and DA (Figure 8.7); fractions collected during the last 10 minutes of perifusion with LHRH–A and DA had no detectable GtH. Upon substitution of the perifusion solutions containing both

Table 8.3. Summary of experimental treatments on pituitary fragments.

Column	Solutions tested	Fractions collected
III & IV[1]	Hanks with BSA[2]	3
	DA (50 nM)	3
	Hanks with BSA	3
	DA (500 nM)	3
	Hanks with BSA	3
V & VI[3]	Hanks with BSA	2
	2 minutes of LHRH–A (10 nM) followed by 3 minutes of Hanks with BSA	1
	Hanks with BSA	7
	DA (500 nM)	2
	2 minutes of LHRH–A (10 nM)+DA (500 nM) followed by 3 minutes of DA (500 nM)	1
	DA (500 nM)	7
	MET[4] (500 nM)	3
	2 minutes of LHRH–A (10 nM)+MET (500 nM) followed by 3 minutes of MET (500 nM)	1
	MET (500 nM)	6

1 Pituitary fragments were prepared from female goldfish in late ovarian recrudescence (June; n=6; GSI=17.5±0.7%). 10 minute fractions were collected.

2 Hepes–Hanks solution supplemented with 0.1% bovine serum albumin.

3 Pituitary fragments were prepared from spermiating male goldfish (June; n=4; GSI not determined). 5 minute fractions were collected.

4 Metoclopramide.

Figure 8.5. Changes in GtH content in perifusate of dispersed pituitary cells during perifusion with NE.

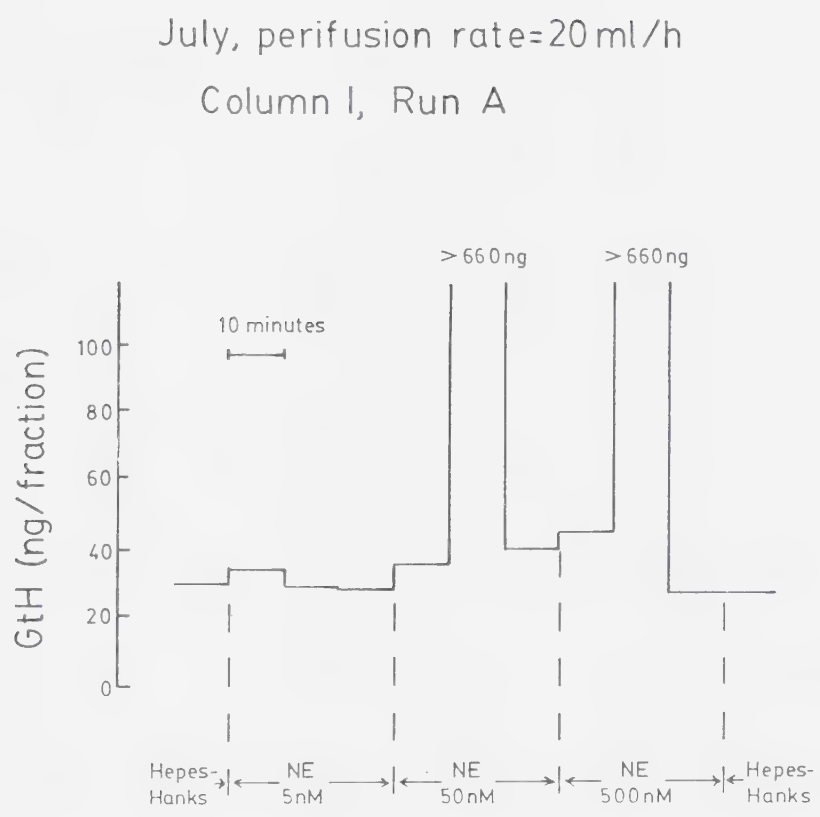


Figure 8.6. Changes in GtH content in perifusate of dispersed pituitary cells during perifusion with DA

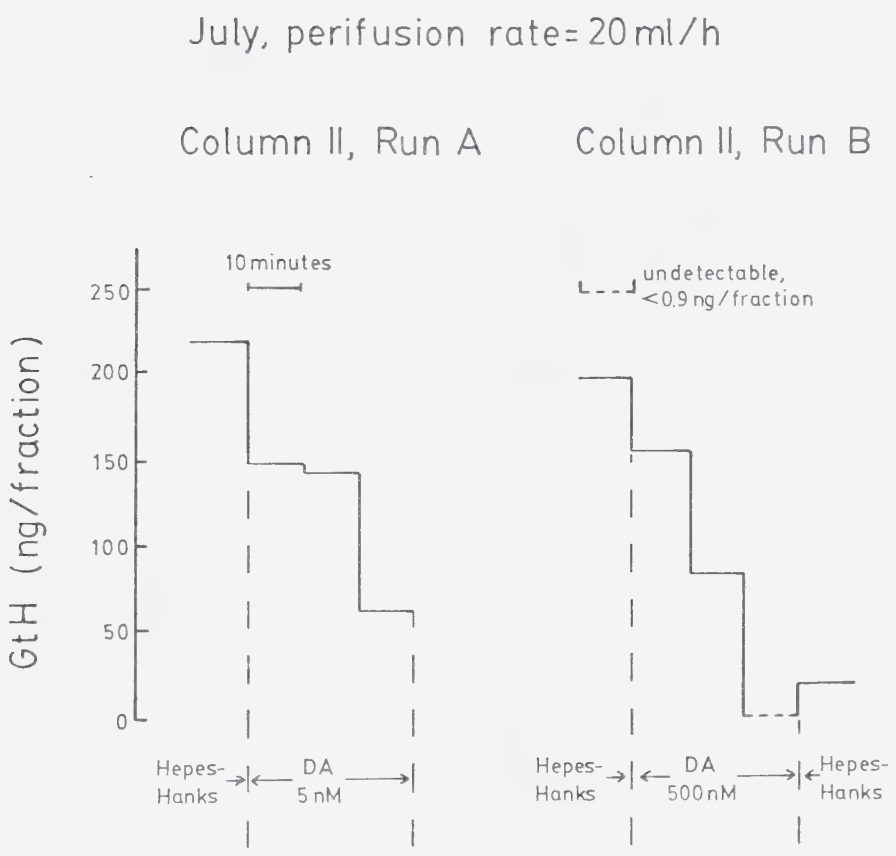
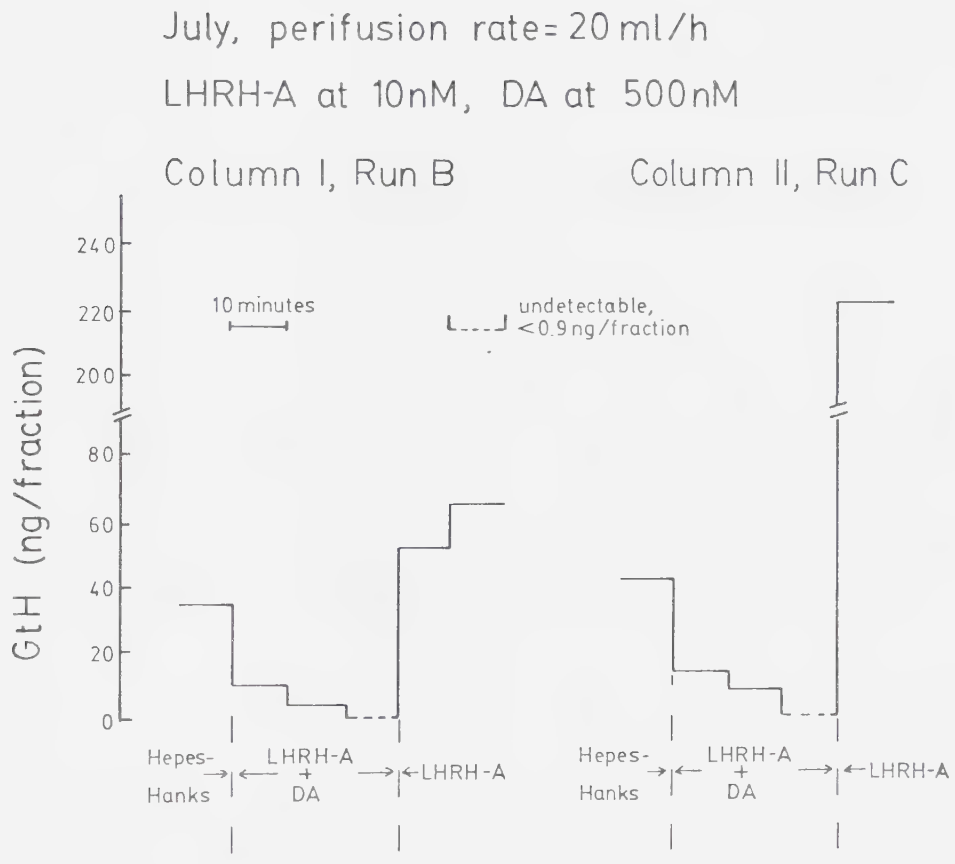


Figure 8.7. Changes in GtH content in perifusate of dispersed pituitary cells during perifusion with LHRH-A and DA.



DA and LHRH–A with ones containing LHRH alone, the GtH content of the perifusates increased from undetectable levels to levels that ranged from 50 to 220 ng/fraction (Figure 8.7).

Pituitary Fragments

The GtH content of fractions collected during perifusion of columns III and IV with DA at 50 and 500 nM were lower than those obtained just before exposure to these concentrations of DA (Figure 8.8). Fractions collected between 20–30 minutes following the termination of perifusion with 50 (column IV) or 500 nM DA (columns III and IV) had higher GtH contents than those obtained during the last 10 minutes of perifusion with these two concentrations of DA (Figure 8.8).

The amount of GtH contained in fractions collected during perifusion of columns V and VI with 500 nM DA were lower than those obtained just prior to exposure to DA (Figure 8.9). Fractions collected by the end of the perifusion with either 500 nM metoclopramide or albumin supplemented Hepes–Hanks solution alone had similar GtH contents (Figure 8.9). Fractions collected between 5–10 minutes following exposure to a 2 minute pulse of 10 nM LHRH–A during perifusion with either albumin supplemented Hepes–Hanks alone or 500 nM metoclopramide, but not during perifusion with 500 nM DA, had higher GtH contents than those obtained just prior to LHRH–A administration (Figure 8.9).

D. Discussion

Dispersed goldfish pituitary cells and pituitary fragments continued to release GtH *in vitro*, as in previous studies (D. MacKenzie, unpublished results). Since the various perifusion solutions do not interfere with the radioimmunoassay measurements of GtH (J. Chang, unpublished results), changes in the GtH content of the perifusates directly reflect alterations in the *in vitro* GtH secretion rate of the gonadotrops.

Perifusion of dispersed pituitary cells harvested from goldfish undergoing or having completed ovarian regression, with 50 and 500 nM of NE increased the amount of GtH in the perifusate (Figure 8.5). These results demonstrate that NE can directly stimulate an increase in basal GtH release *in vitro*, supporting the hypothesis derived from *in vivo*

Figure 8.8. Changes in GtH content in perifusate of pituitary fragments during and following exposure to DA.

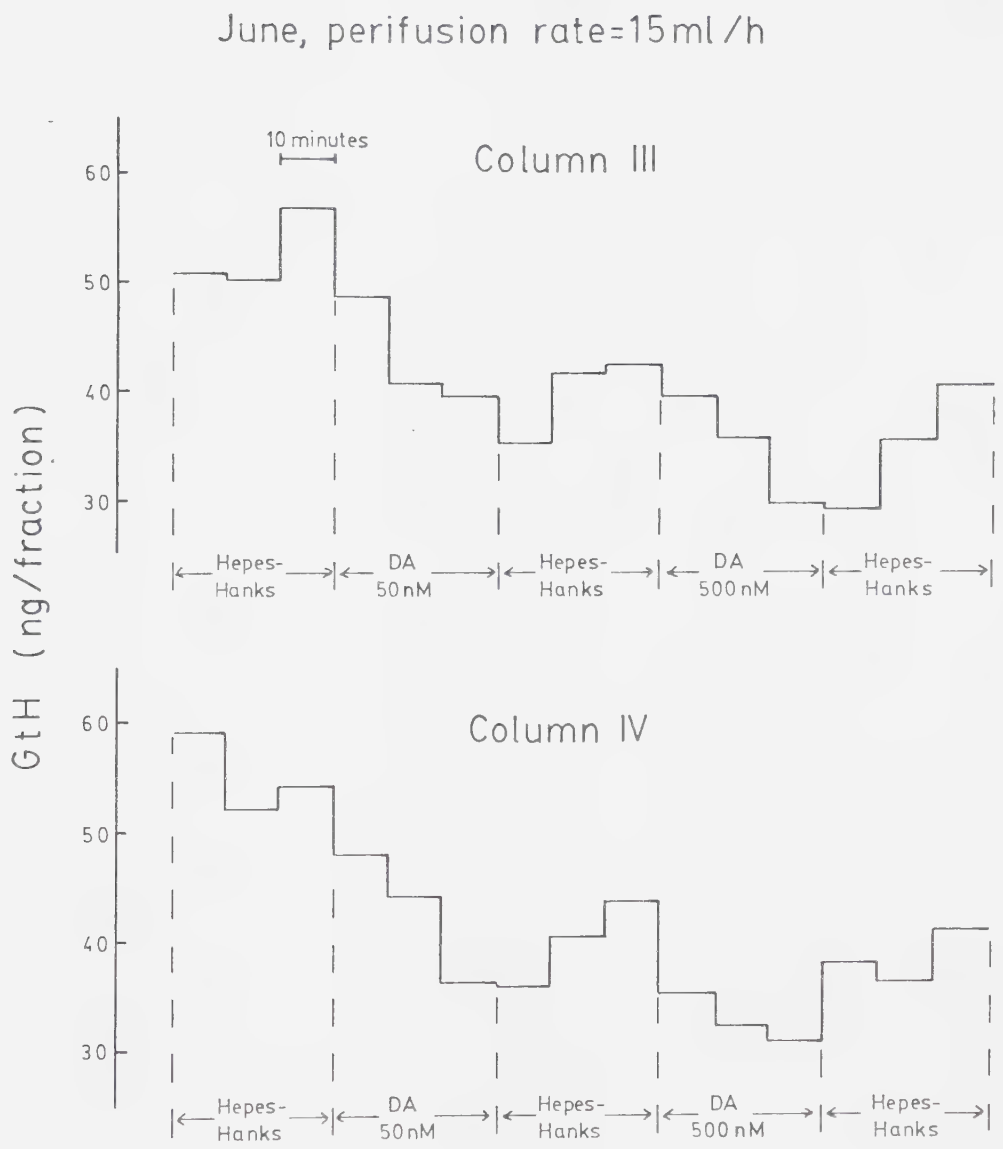
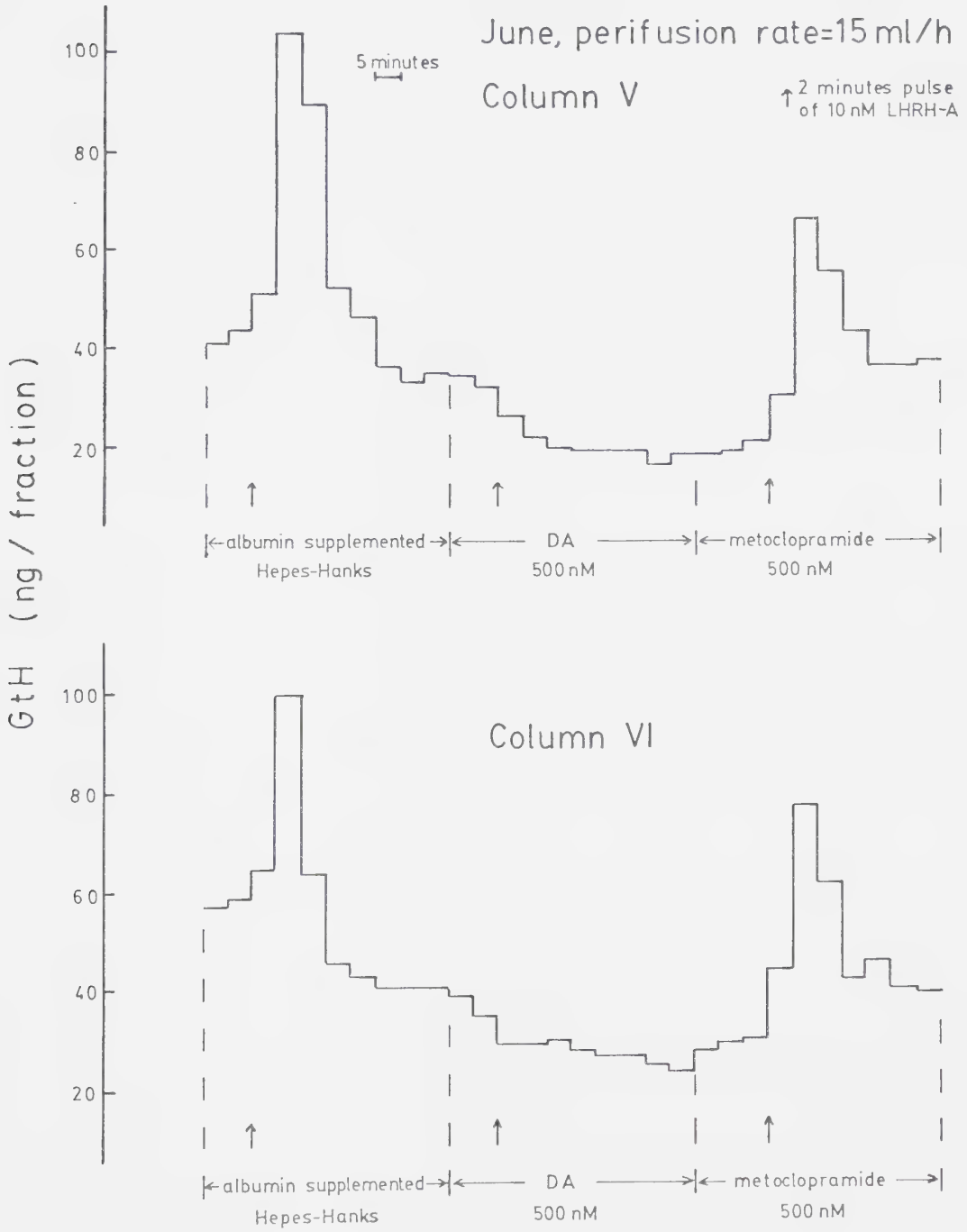


Figure 8.9. Changes in GtH content in perifusate of pituitary fragments in response to a 2 minute pulse of 10 nM LHRH-A during perifusion with either albumin supplemented Hepes-Hanks solution alone, 500 nM DA, or 500 nM metoclopramide.



studies that NE has a direct stimulatory influence on GtH secretion in female goldfish with regressed ovaries. However, these results contradict the findings that NE does not alter LH release from rat pituitries *in vitro* (Kamberi and McCann, 1969; Quijada *et al.*, 1973).

Although NE stimulated *in vitro* GtH release from dispersed goldfish pituitary cells, the GtH release–response was not sustained on continual exposure to the same preparation of NE solution (Figure 8.5). The failure to maintain the GtH–release response was unlikely the result of a depletion of the cellular GtH pool, because exposure to 50 and 500 nM NE, in series, each stimulated GtH release transiently. However, the transient nature of the response may be due to desensitization of the gonadotrops to a constant level of the NE–stimulus and/or the loss of NE from the perifusion solution due to spontaneous oxidation. In mammals, it is well established that down regulation and desensitization of receptors occurs on exposure to a constant high level of stimulus (for review see Catt *et al.*, 1979). It is also well known that catecholamines can undergo spontaneous oxidation on exposure to air.

Perifusion of dispersed goldfish pituitary cells with 5 and 500 nM DA (Figure 8.6) and perifusion of pituitary fragments with 50 and 500 nM DA (Figures 8.8 and 8.9) decreased the GtH content in perifusates. These results indicate that DA decreases spontaneous GtH release *in vitro*, and are consistent with the idea developed from results of *in vivo* studies (Chapters III and IV) that DA acts directly on gonadotrops to inhibit GtH release. However, as described in the General Introduction (Chapter I), DA does not affect LH release from mammalian pituitaries *in vitro*.

In this study, a similar concentration (500 nM) of DA was more effective in suppressing spontaneous GtH release from dispersed pituitary cells than from pituitary fragments (Figures 8.6, 8.8 and 8.9). Since most, if not all, cells would be directly exposed to the perifusion solution in the dispersed cell system, whereas the perifusion medium would not have as direct access to cells located at, or near, the center of the pituitary fragments, the depression of GtH release in response to DA treatment would be expected to be more pronounced with the dispersed pituitary cells than with the pituitary fragments.

After termination of exposure to DA, the GtH content of the perifusates increases immediately in experiments with dispersed pituitary cells (Figure 8.6) and within 20 minutes in experiments with pituitary fragments (Figure 8.8), indicating that the inhibitory effects of

DA on spontaneous GtH release are reversible. The longer latency required for the recovery of normal basal release following DA treatment in experiments with pituitary fragments may be the result of a slower rate of removal of DA from the perifusion system due to the poor accessibility of some of the cells of the pituitary fragments to the perifusion medium. Comparing the GtH content of the perifusates from pituitary fragments collected just before exposure to 500 nM DA to those obtained between 40–50 minutes following the replacement of the DA solutions with ones containing 500 nM metoclopramide (Figure 8.9), it is apparent that the recovery of the spontaneous GtH release rate to a normal level had occurred by 50 minutes following termination of perifusion with DA.

In the present investigation, 10 nM LHRH–A increased the GtH release from perifused goldfish pituitary cells (Figure 8.7) and pituitary fragments (Figure 8.9). The ability of LHRH and its analogues to stimulate LH release from mammalian pituitaries *in vitro* is well documented (for review see Catt *et al.*, 1983). LHRH–A and LHRH have been shown to stimulate GtH secretion *in vitro* from pituitaries of rainbow trout (Crim and Evans, 1980; Crim *et al.*, 1981), grass carp (Pan *et al.*, 1980), and brown trout (Crim *et al.*, 1981). Perifusion of 500 nM DA at the time of exposure to 10 nM LHRH–A abolished the *in vitro* GtH release–response to the peptide in both the dispersed goldfish pituitary cells (Figure 8.7) and pituitary fragments (Figure 8.9). These results are consistent with the hypothesis developed from *in vivo* experiments (Chapters III, V and VI) that DA directly interferes with GnRH actions in goldfish. DA has been shown to interfere with the actions of LHRH in rabbits (Dailey *et al.*, 1978) and humans (Judd *et al.*, 1978; Huseman *et al.*, 1980).

To examine if previous exposure to DA caused a permanent change in the response of pituitary fragments to LHRH–A, a 500 nM metoclopramide solution was perifused over the fragments upon termination of DA treatment before testing with LHRH–A. This was done to displace DA from its receptors and to ensure that no DA receptor would be available to the DA molecules, thereby terminating the actions of DA as rapidly as possible. A 2 minute pulse of LHRH–A increased GtH release from the pituitary fragments during perifusion with metoclopramide, although no release–response was observed during perifusion with DA (Figure 8.9). This indicates that the DA blockage of

GnRH actions on pituitary fragments is reversible. The DA blockage of GnRH actions in the dispersed pituitary cells perifusion system also appeared to be reversible, as the amount of GtH released from the dispersed cells increased immediately upon replacing the perifusion solution from one containing both DA and LHRH-A to one containing LHRH-A alone (Figure 8.7).

The magnitudes of the *in vitro* GtH release-response of pituitary fragments to a 2 minute pulse of 10 nM LHRH-A alone or with 500 nM metoclopramide were similar, both groups showing increases of 40-60 ng in the GtH content of perifusates within 10 minutes of exposure to LHRH-A (Figure 8.9). However, in *in vivo* studies metoclopramide greatly potentiated the ability of LHRH-A to increase goldfish serum GtH concentrations (Chapter VI). These results indicate that metoclopramide has no inherent ability to potentiate LHRH-A-induced GtH release, but is effective *in vivo* due to its ability to block the actions DA.

In experiments presented in this chapter, the maximal GtH responses to the various drug treatments were usually only reached some time after the commencement of drug perifusion (Figures 8.5 to 8.9). This delay may reflect a problem with the dilution of drug solutions within the dead-space in the columns above the dispersed pituitary cells and pituitary fragments. The delay may also be caused, in part, by the response latency of the gonadotrops to the different drug solutions.

Results from this study suggest that the minimal effective dose of NE required to stimulate GtH release from goldfish gonadotrops *in vitro* is between 5 and 50 nM (Figure 8.4). The minimal dose of DA required to inhibit GtH release from goldfish gonadotrops *in vitro* would be less than or equal to 5 nM, as 5 nM DA, the lowest concentration of DA tested, was effective in decreasing spontaneous GtH release (Figure 8.5). The amount of DA and NE present in the goldfish pituitary has been estimated at approximately 85 and 110 pg/pituitary (n=13), respectively (J. Chang, unpublished results). Assuming that the average wet weight of goldfish pituitary is 1.5 mg (average of 8 separate determinations; J. Chang and R. Peter, unpublished data) and that the specific gravity of the pituitary is close to unity, the concentration of DA and NE in the goldfish pituitary would both be approximately 400 nM. This suggests that the minimal effective dosages of DA and NE found to alter spontaneous GtH release *in vitro* would be within the probable

physiological ranges that the gonadotrops would be exposed to *in vivo*.

In summary, results from the present study provided *in vitro* evidence for the presence of direct DA inhibition of spontaneous GtH release, DA blockage of GnRH actions, and NE stimulation of GtH release in goldfish.

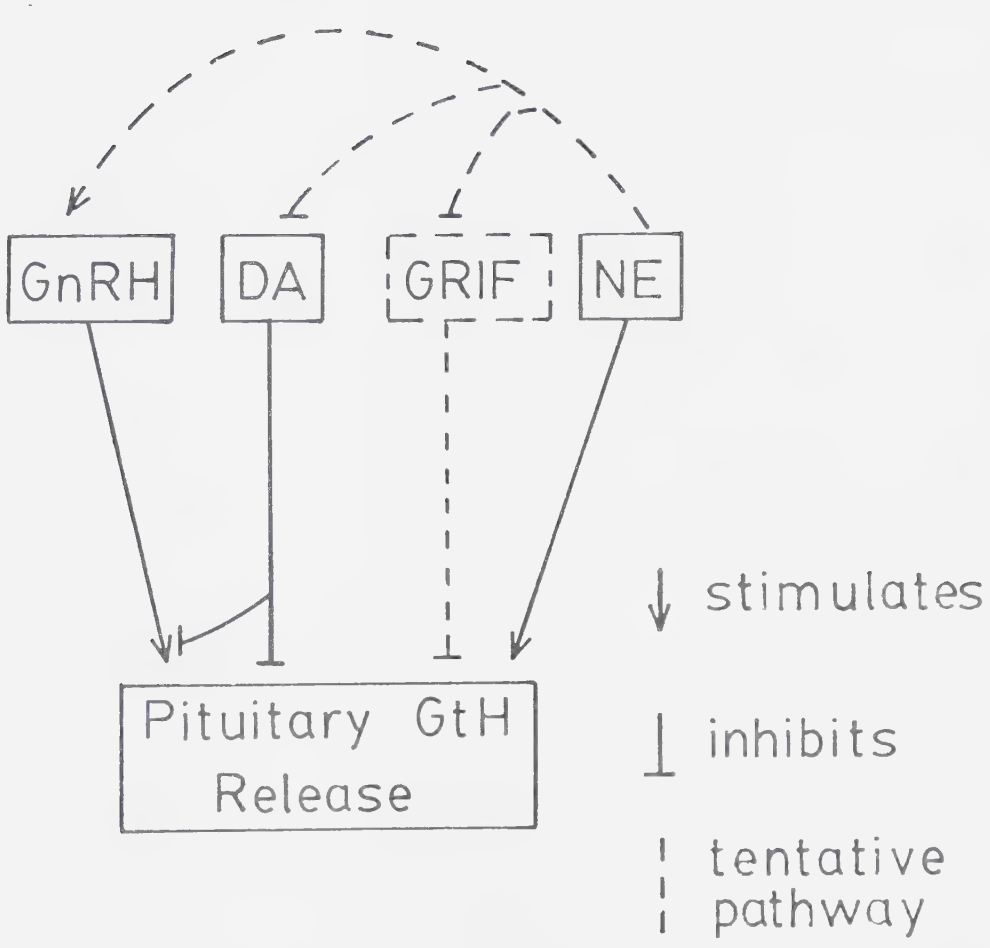
IX. General Discussion

In previous chapters, new information regarding the influences of DA and NE on GtH release in goldfish were presented. A brief summary and an overall discussion of these experimental findings are presented in this chapter.

Results from *in vivo* experiments with drugs capable of blocking specific enzymes involved in catecholamine synthesis suggest the existence of an inhibitory influence of DA on GtH release in goldfish (Chapter II). In two preliminary experiments, intraperitoneal injections of L-DOPA (the precursor to DA) were found to decrease serum GtH levels (Appendix III). Intraperitoneal injections of DA and apomorphine decreased (Chapter III), whereas pimozide (Chapters III, V and VI) and metoclopramide (Chapter VI) increased serum GtH concentrations in goldfish. These results are consistent with the hypothesis that DA inhibits GtH release in goldfish. In mammals, the existence of a blood-brain barrier for DA is well known. A blood-brain barrier for DA probably exists in the goldfish since systemically administered radio-labelled DA was not taken up readily by brain tissues (J. Chang and R. Peter, unpublished results). Since the pituitary lies outside of the blood-brain barrier, the results with intraperitoneal injections of DA also suggest that DA acts at the level of the pituitary to inhibit GtH release. Direct innervation of the gonadotrops in goldfish has been reported (Chapter I). The failure of intraventricular injections of DA to alter serum GtH levels (Chapter III) also lends support to the idea that the influences of DA on GtH release in goldfish are due to actions directly on the pituitary.

As discussed in Chapters III and IV, goldfish bearing preoptic lesions or pars distalis transplants are useful models for studying the effects of DA on spontaneous GtH release *in vivo*. Intraperitoneal injections of DA or apomorphine each reduced the elevated serum GtH levels in preoptic lesioned female goldfish (Chapter III) and in male goldfish bearing pars distalis transplants (Chapter IV). These results, together with the observation that perifusion of dispersed goldfish pituitary cells or pituitary fragments with DA decreases the GtH content of perifusates (Chapter VIII), indicate that DA directly inhibits the spontaneous release of GtH in goldfish (Figure 9.1). These findings are in direct contrast to the idea developed from studies on mammals, described in Chapter I, that DA alters LH release by actions on LHRH secretion.

Figure 9.1. Summary diagram of the influences of DA and NE on GtH release.



The influences of DA, and its agonists and antagonists on the actions of LHRH-A were studied to determine the possible interactions between DA and GnRH. Intraperitoneal injections of DA (Chapter III), apomorphine (Chapters III and VI) and bromocriptine (Chapter VI) reduced, and pimozide (Chapters V and VI) and metoclopramide (Chapter VI) potentiated the LHRH-A induced increase in serum GtH levels in goldfish. Perifusion of dispersed goldfish pituitary cells or pituitary fragments with DA abolished the *in vitro* GtH release-response to LHRH-A treatments (Chapter VIII). Together, these results suggest that DA directly inhibits GnRH actions in goldfish (Figure 9.1). As discussed in Chapter I, DA has been shown to inhibit the actions of injected LHRH in humans and rabbits.

Results with intraperitoneal injections with clonidine (Chapters II and VII; Appendix III), phentolamine, propranolol, and octopamine (Chapter VII) suggest that the DA inhibition on GtH secretion is specific. The results with intraperitoneal injections of metoclopramide, pimozide, apomorphine and bromocriptine (Chapters III to VI) suggest that DA acts via D-2 receptors to inhibit GtH release. In mammals, DA binding to D-2 receptors has been shown to inhibit cAMP accumulation in anterior pituitaries (for review see Creese *et al.*, 1981; Creese, 1982). Bromocriptine and apomorphine each mimic (Onali *et al.*, 1981), whereas pimozide and metoclopramide each reverse (Meunier and Labrie, 1982) the DA inhibition of adenyl cyclase activity in rat pituitary glands. Although results from recent investigations in mammals demonstrate that Ca^{2+} mobilization rather than cAMP accumulation was the immediate cellular response of gonadotrops to LHRH and its analogues, findings from earlier experiments showed that cAMP mediates LHRH stimulation of LH release (for reviews see Clayton and Catt., 1981; Conn *et al.*, 1981). Since Ca^{2+} and its intracellular receptor, calmodulin, regulate both phosphodiesterase and adenyl cyclase activities (for review see Means and Chafouleas, 1982), and the Ca^{2+}-calmodulin and adenyl cyclase-cAMP systems have been shown to interact in the regulation of prolactin secretion in rats (Schettini *et al.*, 1983), it is possible that LHRH actions are also mediated by interactions of the two "second messengers", Ca^{2+} and cAMP. In goldfish, DA may act through sites that resemble the D-2 receptors to directly inhibit GtH release and GnRH actions by decreasing adenyl cyclase activity and cAMP accumulation.

The ability of DA to inhibit GtH release is apparently not restricted to goldfish, but is also present in other cyprinids, and in catostomid and salmonid species. Intraperitoneal injections of pimozide increased serum GtH concentrations in the common carp (Sokolowska *et al*, unpublished results). In the white sucker, intraperitoneal injections of pimozide increased and apomorphine decreased serum GtH concentrations (Appendix IV). Crim (1981) reported in an abstract that DA inhibited basal and LHRH stimulated GtH release from rainbow trout pituitaries *in vitro*. In coho salmon, intraperitoneal injections of pimozide not only increased normal serum GtH levels but also potentiated LHRH-A stimulated GtH release (G. Van Der Kraak and J. Chang, unpublished results).

In Chapters V and VI, it was observed that injections of LHRH-A alone stimulated GtH release, but failed to induce ovulation in a significant number of gravid female goldfish kept at 12°C; injections of pimozide or metoclopramide not only potentiated the LHRH-A induced increase in serum GtH levels to levels similar to those found in spontaneously ovulating goldfish, but also increased the frequency of occurrence of ovulation. These results suggest that the preovulatory surge of GtH release in goldfish is regulated by both stimulation by GnRH and removal of DA inhibition on GtH release. The idea that the removal of DA inhibition of GtH secretion is part of the mechanism regulating the preovulatory release of GtH is the first time a physiological role has ever been assigned to DA in the regulation of GtH release in fish.

The finding that blockage of the DA inhibition by pimozide or metoclopramide increased the chance of successfully inducing ovulation in goldfish by LHRH-A injections (Chapters V and VI) has potential application in aquaculture. A report from France (Billard *et al*., 1983) indicated that pimozide injection potentiated the ability of a LHRH analogue ($D\text{-}Trp^6$) to stimulate GtH release in male and female common carp; and this combined treatment was useful in inducing spermiation, and oocyte maturation and ovulation in common carp. Pimozide injections were reported to increase the ability of LHRH-A to induce ovulation in carp in Poland (K. Bieniarz and R. Peter, unpublished results) and in several cultured fish species in China, including the mud carp, black carp, silver carp, and the roach (H. R. Lin and C. H. Pan, personal communication).

The results discussed above indicate clearly that DA has GRIF activity in goldfish. Based on results from brain lesioning experiments, Peter and Paulencu (1980) concluded

that, in goldfish, GRIF originates from the anterior ventral portions of the nucleus preopticus periventricularis, and that bilateral nerve tracts carrying GRIF project caudally from these locations towards the pituitary via the lateral preoptic and lateral tuberal regions of the hypothalamus. DA cell bodies were recently identified in the anterior ventral preoptic area of the goldfish by immunohistochemical techniques, and the DA neurons were found to project to the pituitary via bilateral tracts in the preoptic and tuberal hypothalamus (O. Kah, personal communication). Lesions that interrupted the neural pathway carrying GRIF caused degeneration of immunohistochemically identifiable DA terminals adjacent to gonadotrops in the pituitary of goldfish (O. Kah and R. Peter, personal communication). These results, together with the finding that intraperitoneal injection of DA decreased the elevated serum GtH levels caused by preoptic lesions (Chapter III), indicate that DA is indeed the GRIF in goldfish. Although it is possible that other factors may also have GRIF activity in goldfish, DA clearly is prominent in this regard.

Results with intraperitoneal injections of NE (Chapter VII) suggest that NE directly stimulates GtH release in goldfish at times of year when the ovaries are regressed or undergoing early stages of recrudescence (Figure 9.1). The ability of NE to directly stimulate GtH release was confirmed by *in vitro* experiments using dispersed goldfish pituitary cells (Chapter VIII). Since the ability of intraperitoneal injections of clonidine to increase serum GtH levels at different times of the year parallelled that of intraperitoneally injected NE (Chapter II and VII; Appendix III), and phentolamine blocked the NE induced increase in serum GtH levels (Chapter VII), it is concluded that NE stimulates GtH release via alpha-receptors. In mammals, the effects of stimulation of alpha-receptors are mediated by the Ca^{2+}-calmodulin system (for review see Means and Chafouleas, 1982). Since the LHRH stimulation of LH release involves redistribution of Ca^{2+} (for review see Clayton and Catt, 1981; Conn *et al.*, 1981; Catt *et al.*, 1983), it is likely that NE, via alpha-receptors, stimulates Ca^{2+} influx into the gonadotrops, thereby causing an increase in GtH release.

Although in goldfish, the gonadotrops are directly innervated (see Chapter I) and results described in the previous paragraph suggest that NE directly stimulates GtH release, it is possible that NE may also act through central sites to increase GtH secretion. In support of this latter possibility intraventricular injection of NE was found to increase serum GtH levels (Chapter VII). In Chapter VII, it was proposed that NE may stimulate GnRH

secretion to increase GtH release, as in mammals. It is also possible that the central action of NE is to decrease the secretion of GRIF (Figure 9.1).

As described in Chapter I, NE modulates the preovulatory LH surge in mammals. The physiological role that NE may have in the neuroendocrine regulation of GtH release in goldfish is not known. However, NE probably is not involved in the regulation of the preovulatory GtH release in fish since NE injections had no effect on serum GtH levels in female goldfish with matured ovaries or ovaries at later stages of recrudescence (Chapter VII).

As discussed in Chapter VII, the stimulatory influence of NE on GtH release seems to be seasonal. The basis for the seasonality of the influence of NE on GtH secretion is not known. In Chapter VII, it was suggested that seasonal changes in steroid levels may influence the GtH release–response to NE. Schreck and Hopwood (1974) reported that serum sex steroid levels in female goldfish increased with the degree of maturation of the ovaries, with the highest circulating levels of estrogen occuring at the spawning season. L. Garcia (personal communication) demonstrated that estradiol production from goldfish ovaries increased with the maturation of the oocytes. Estradiol–17–beta and estrone have been shown to inhibit H^3–NE binding to membrane fragments of rat brain in a dose dependent manner (Inaba and Kamata, 1979). The number of DA binding sites in the rat anterior pituitary has been shown to be decreased by estradiol treatment (Heiman and Ben–Jonathan, 1982a, b). It is possible that the high serum sex steroid levels, found in female goldfish with matured ovaries or ovaries at later stages of recrudescence, inhibits the binding of NE to receptor sites in the pituitary and the brain, and/or decreases the number of these binding sites, thereby eliminating the GtH release–response to NE in goldfish at these particular stages of the ovarian cycle.

Although the stimulatory influence of NE on GtH release is seasonal (Chapter VII), DA has the ability to inhibit GtH release throughout the reproductive cycle of the goldfish. The ability of DA or its agonists to decrease GtH release was demonstrated *in vivo* using goldfish with matured gonads or gonads at early stages of recrudescence (Chapter III and IV). The ability of DA to decrease the GtH release *in vitro* was demonstrated with pituitaries obtained from fish having matured or regressed gonads (Chapter VIII). The effectiveness of injections of DA antagonists to potentiate the LHRH–A stimulated GtH

release was demonstrated in fish with matured gonads as well as in fish at other stages of gonadal recrudescence cycle (Chapters V and VI). The balance between various stimulatory influences and the DA inhibition on GtH release is probably important in determining the rate of GtH secretion throughout the entire reproductive cycle of the goldfish.

In summary, results from experiments contained in this thesis indicate that DA has direct GRIF activity on gonadotrops in goldfish to inhibit spontaneous GtH release and to block GnRH actions (Figure 9.1). The results also suggest that the preovulatory GtH surge may be regulated by both a stimulation by GnRH and by release from the DA inhibition on GtH secretion. The DA antagonists, pimozide and metoclopramide, in combination with LHRH or its analogues may be useful tools for increasing the success of inducing ovulation in fish in aquaculture. Results from this study also indicate that NE stimulates GtH release in goldfish by alpha-adrenergic mechanisms at the pituitary and/or brain level in female goldfish in a sexually regressed or early ovarian recrudescence condition (Figure 9.1).

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Appendix I

GENERAL AND COMPARATIVE ENDOCRINOLOGY 49, 22–31 (1983)

Influence of Catecholamines on Gonadotropin Secretion in Goldfish, *Carassius auratus*

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Serum gonadotropin (GtH) concentrations in female goldfish were measured before and at various times after intraperitoneal injection of drugs altering catecholamine synthesis and neural activities. Reserpine, a depleter of neurotransmitter stores, elevated serum GtH levels compared to controls, suggesting the general involvement of neurotransmitters in altering GtH release. 6-Hydroxydopamine, a catecholaminergic neurotoxin, increased serum GtH concentration, suggesting that catecholaminergic neurons inhibit GtH release. Blocking of L-DOPA and dopamine synthesis by α-methyl-*para*-tyrosine and carbidopa, respectively, but not norepinephrine by diethyldithiocarbamate, raised serum GtH values above those of controls. Injections of an α-agonist, clonidine, also increased serum GtH concentrations. These results suggest the existence of an inhibitory dopaminergic and a stimulatory α-adrenergic influence on GtH release in goldfish.

Studies on various mammalian species have shown that certain drugs that alter brain neurotransmitter activity can influence the secretion of luteinizing hormone (LH) (for review see Sawyer, 1979; Krulich, 1979). Dopamine (DA) has been found to both stimulate and inhibit LH secretion, whereas norepinephrine (NE) has more consistently been found to stimulate LH release (for review see Sawyer, 1979; McCann, 1980). Both catecholamines are presumed to affect LH secretion by altering the release of luteinizing hormone-releasing hormone (LHRH) (Krulich, 1979; Sawyer, 1979; Gallo, 1980; McCann, 1980; Porter *et al.*, 1980); however, DA has, in some cases, been shown to directly inhibit the action of LHRH (Dailey *et al.*, 1978; Judd *et al.*, 1978).

Hypothalamic neurosecretory neurons terminate in the median eminence in mammals, but in teleosts the median eminence is rudimentary at best, and at least two types of morphologically distinct hypothalamic neurons directly innervate the pars distalis (for review see Peter and Crim, 1979). These two fiber types, considered to represent peptidergic and aminergic fibers respectively, have been shown to innervate gonadotrophs of many teleosts, including the goldfish (Leatherland, 1972; Kaul and Vollrath, 1974). This provides the possibility that gonadotropin (GtH) release in fish can be regulated by the actions of hypothalamic hormones and/or direct action of neurotransmitter agents on gonadotrophs. The presence in teleosts of a gonadotropin releasing hormone (GnRH) that is similar to LHRH has been demonstrated (for review see Peter, 1982). In addition, Peter and Crim (1978) and Peter and Paulencu (1980) have suggested the presence of a gonadotropin release-inhibitory factor (GRIF) in goldfish on the basis of brain lesioning experiments. However, there are relatively few data available on the actions that neurotransmitter agents may have in the regulation of GtH secretion in fish. Microscopic examination revealed that *in vivo* treatment with reserpine enhanced the activities of gonadotrophs in the black molly and medaka (Egami and Ishii, 1962; Sage and Bromage, 1970). Epinephrine and NE enhanced the *in vitro* adenyl cyclase activity of the pars distalis of goldfish (Deery, 1975). More recently, Crim (1982) demonstrated that DA inhibited the

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basal and LHRH-stimulated GtH release from the pituitary of rainbow trout *in vitro*. These data suggest that neurotransmitters may have direct as well as indirect actions, via alteration of the secretion of hypothalamic hormones, on gonadotrophs and GtH secretion in teleosts.

In the present study, the possible involvement of neurotransmitters in regulation of GtH secretion in female goldfish, *Carassius auratus*, was investigated by determining the effects on serum GtH levels of intraperitoneal injection of drugs that affect catecholamine synthesis and neural activities.

MATERIALS AND METHODS

General

Goldfish (comet or common variety), purchased from Grassyforks Fisheries Company, Martinsville, Indiana, were held on a 12-hr light:12-hr dark (12L:12D; lights on at 0800) photoperiod in flow-through aquaria (150 or 225 liters) at 12° for at least 7 days after arrival. Seven days prior to an experiment, the photoperiod was usually extended to 16L:8D. Fish were fed daily, *ad libitum*, with Ewos trout pellets.

Reserpine, 6-hydroxydopamine HCl (6-OHDA), α-methyl-*para*-tyrosine methyl ester HCl (αMPT), and diethyldithiocarbamate sodium salt (DDC) were purchased from Sigma, St. Louis, Missouri. Clonidine HCl (Catapres, clonidine) and carbidopa were gifts from Boehringer–Ingelheim Ltd., Ridgefield, Connecticut, and Merck Frosst Laboratories, Dorval, Quebec, Canada, respectively. The drugs (dosages given below) were made up fresh before injection in a vehicle of acidified 0.7% NaCl with 0.1% sodium metabisulfite, and injected intraperitoneally as either a solution or suspension. Control groups were given an equivalent volume of vehicle.

Fish were anesthetized with tricaine methanesulfonate (Sigma, 0.03 to 0.05%) before handling. Prior to each experiment, the fish were weighed and identified with a numbered metal tag attached to the operculum. Blood of each fish was sampled serially by puncturing the caudal vasculature with a 25-gauge, 0.5-in. needle attached to a 1.0-ml disposable syringe. Blood samples were allowed to clot on ice for several hours, and the serum was separated by centrifugation and stored at −28°. Serum GtH levels were determined by radioimmunoassay (Crim *et al.*, 1976; Hontela and Peter, 1978, 1980). After each experiment, the fish were killed by decapitation, and the body and gonads weighed for determination of gonadosomatic index (GSI). The ovaries were also categorized as being in a regressed or inactive condition if the GSI was below 1%, in an early recrudescing state if they appeared grayish-green (GSI between 1 and 3%), in midrecrudescence if they appeared grayish-green and contained small (barely visible to the naked eye) vitellogenic oocytes (GSI between 3 and 8%), in late recrudescence if they contained large, white vitellogenic oocytes (GSI between 8 and 16%), in final maturation if the oocytes had completed vitellogenic growth (GSI above 16%), and in a regressing state if the ovaries contained a large number of yellow, atretic oocytes.

Signed-ranks test or paired *t* test was used to compare pretreatment and posttreatment serum GtH levels within experimental groups. The Mann–Whitney *U* test was used to compare experimental and control GtH concentrations (Snedecor and Cochran, 1971). When the pretreatment serum GtH levels of the control and experimental groups were significantly different (*U* test), GtH values were expressed as a percentage of the pretreatment value before comparisons by *U* tests were made.

Experimental treatments

The details of the various experimental groups and treatments are summarized in Table 1.

RESULTS

In female goldfish that were in early or midgonadal recrudescence, reserpine caused a significant increase in serum GtH concentrations above those of controls at 24 hr but not at 6 hr postinjection (Table 2). In female goldfish undergoing gonadal regression, as well as those in late gonadal recrudescence or final gonadal maturation, 6-OHDA injections caused a significant elevation in serum GtH levels 1 week after the initiation of drug treatment when compared to controls (Table 3); however, the 6-OHDA-induced increase in serum GtH concentration was not observed at other sampling times during the 3-week treatment period (Table 3). In fish in early ovarian recrudescence, αMPT injections prevented the decrease in serum GtH levels observed in the controls at 6 and 24 hr postinjection (Table 4). In goldfish in early recrudescence, αMPT caused a consistent elevation in serum GtH levels at 24 hr postinjection compared to controls when the results are expressed as percentage of the pretreat-

TABLE 1
SUMMARY OF EXPERIMENTAL GROUPS

Treatment	Date	Gonadal condition	GSI	Sample times (hr postinjection)
Reserpine (100 μg/g)[a]	Feb. 1980	Midrecrudescence	3.3 ± 0.5%[b]	0,[c] 6, 24
	Oct. 1980	Early recrudescence	2.2 ± 0.2%	0, 6, 24
6-OHDA[d] (50 μg/g, twice weekly)	June–July 1980	Regressing/regressed	3.4 ± 1.0%	2 hr, 1, 2, 3 weeks[e]
	Apr. 1981	Late recrudescence/ final maturation	9.9 ± 0.9%	1, 3 weeks[e]
αMPT (5 μg/g, Nov. 1979; 300 μg/g, Apr. and Oct. 1980)	Nov. 1979	Early recrudescence	2.8 ± 0.4%	0, 6, 24
	Oct. 1980	Early recrudescence	2.5 ± 0.5%	0, 6, 24
	Apr. 1980	Late recrudescence	9.4 ± 1.1%	0, 6, 24
Carbidopa (50 μg/g)	Feb. 1980	Midrecrudescence	3.8 ± 0.5%	0, 6, 24
	Oct. 1980	Early recrudescence	2.1 ± 0.1%	0, 6, 24
DDC (2.5 μg/g)	Feb. 1980	Midrecrudescence	3.8 ± 0.6%	0, 6, 24
	June 1980	Regressing	5.7 ± 0.6%	0, 6, 24
Clonidine (30 μg/g)	Feb. 1980	Midrecrudescence	4.1 ± 0.5%	0, 6, 24
	Oct. 1980	Early recrudescence	2.0 ± 0.3%	0, 6, 24

[a] Doses as μg/g body wt.
[b] Mean ± SE.
[c] Sampled just prior to drug or vehicle injection.
[d] The photoperiod was not extended to 16L:8D for 6-OHDA experiments.
[e] Times after initial injection.

ment value (Table 4). In comparison with pretreatment serum GtH levels, and also using the percent of the pretreatment value to express the results, αMPT injection also caused a significant elevation in serum GtH values at 6 hr postinjection in female goldfish in late gonadal recrudescence; at 24 hr postinjection serum GtH levels were significantly elevated compared to pretreatment levels but not in comparison to controls. Injections of carbidopa into goldfish in either early or midovarian recrudescence caused a significantly higher serum GtH level than in controls at 24 hr postinjection when the results were expressed as the percent of pretreatment

TABLE 2
EFFECTS OF INTRAPERITONEAL INJECTION OF RESERPINE (100 μg/g) ON SERUM GtH CONCENTRATIONS IN FEMALE GOLDFISH (16L:8D, 12°)

		GtH (ng/ml)		
Gonadal condition	Treatment	0 hr	6 hr	24 hr
Early recrudescence Oct. 1980	Control (n = 7)	2.64 ± 0.59[a]	1.58 ± 0.49[b]	1.01 ± 0.11[b]
	Reserpine (n = 8)	1.44 ± 0.07	1.42 ± 0.21	3.19 ± 0.62[b,c]
Midrecrudescence Feb. 1980	Control (n = 6)	3.65 ± 1.54	13.93 ± 6.54[b]	8.28 ± 2.79[b]
	Reserpine (n = 7)	3.54 ± 0.57	7.89 ± 0.78[b]	28.74 ± 14.37[b,d]

[a] Mean ± SE.
[b] Significantly different from 0 hr value, $P < 0.05$ (two-sided signed-ranks test).
[c] $P < 0.002$ (two-tailed U test) compared to control.
[d] $P < 0.01$ (two-tailed U test) compared to control.

TABLE 3
EFFECTS OF INTRAPERITONEAL INJECTION OF 6-OHDA (50 μg/g, TWICE WEEKLY) ON SERUM GtH CONCENTRATIONS IN FEMALE GOLDFISH (12L:12D, 12°)

Gonadal condition	Treatment	GtH (ng/ml)			
		2 hr	1 week	2 weeks	3 weeks
Regressing or regressed June to July 1980	Control (n = 9, 2 hr; n = 12, 1 week; n = 11, 2 and 3 weeks)	1.05 ± 0.19[a]	1.55 ± 0.40	1.19 ± 0.23	1.72 ± 0.32
	6-OHDA (n = 9, 2 hr and 1 week; n = 7, 2 weeks; n = 4, 3 weeks)	2.18 ± 0.52	4.18 ± 1.59[b]	2.34 ± 0.71	3.18 ± 0.77
Late recrudescence or final maturation Apr. 1981	Control (n = 19, 1 week; n = 14, 3 weeks)	—	19.16 ± 1.66	—	16.45 ± 1.65
	6-OHDA (n = 17, 1 week; n = 14, 3 weeks)	—	29.62 ± 3.41[c]	—	26.03 ± 8.35

[a] Mean ± SE.
[b] $P < 0.05$ (two-tailed U test) compared to control.
[c] $P < 0.01$ (two-tailed U test) compared to control.

value (Table 5). Administration of DDC did not alter serum GtH concentrations relative to controls (Table 6). Injection of clonidine prevented the depression of circulating GtH levels in control female goldfish in early stages of ovarian recrudescence, and elevated serum GtH concentrations above those of controls in females in midovarian recrudescence (Table 7).

DISCUSSION

Results in the present report are consistent with the idea that GtH secretion in fish may be regulated, at least in part, by the action of neurotransmitters. Reserpine depletes neurotransmitter stores by blocking the transport of neurotransmitters into intragranular stores (Gilman *et al.*, 1980). Its effectiveness in teleosts on, specifically, DA and NE stores has been demonstrated by Zambrano (1975). The reserpine-induced increase in serum GtH (Table 3) substantiates earlier morphological observations of increased activity of gonadotrophs following treatment with this drug (Egami and Ishii, 1962; Sage and Bromage, 1970). The present results with reserpine indicate that neurotransmitters, in general, can alter the activities of gonadotrophs, and at least one of these neurotransmitters, directly or indirectly, inhibits GtH secretion in the goldfish. The time required for reserpine to cause a significant increase in goldfish serum GtH (Table 1) is similar to that required for reserpine to cause a similar effect in mammals; complete blockage of the electrically stimulated release of GtH in rats was observed 18–20 hr after reserpine treatment (Rubinstein and Sawyer, 1970) and the maximal depletion of catecholamine stores occurred at 24 hr after drug administration (Gilman *et al.*, 1980).

6-OHDA acts as a neurotoxin and destroys catecholaminergic neurons in mammals as well as in teleosts (Kostrzewa and Jacobowitz, 1974; Zambrano, 1975). The observed increase in serum GtH follow-

TABLE 4
EFFECTS OF INTRAPERITONEAL INJECTION OF αMPT ON SERUM GtH CONCENTRATIONS IN FEMALE GOLDFISH (16L:8D, 12°)

Gonadal condition	Treatment	GtH at times postinjection				
		0 hr	6 hr		24 hr	
		ng/ml	ng/ml	% 0 hr	ng/ml	% 0 hr
Early recrudescence Nov. 1979	Control (n = 4)	4.12 ± 0.74[a]	2.05 ± 0.53[b]	45.85 ± 12.17	2.31 ± 0.66[b]	60.97 ± 21.34
	αMPT (5 μg/g; n = 8)	3.51 ± 0.96	4.57 ± 1.78[c]	146.55 ± 14.73[d]	4.78 ± 0.61[b,e]	175.06 ± 29.09[c]
Early recrudescence Oct. 1980	Control (n = 7, 0 and 24 hr; n = 6, 6 hr)	3.05 ± 1.35	1.47 ± 0.24[b]	69.87 ± 12.57	1.25 ± 0.19[b]	58.63 ± 7.40
	αMPT (300 μg/g; n = 5)	1.67 ± 0.19	1.44 ± 0.15	88.98 ± 9.92	1.90 ± 0.29	116.69 ± 16.21[d]
Late recrudescence Apr. 1980	Control (n = 7)	2.99 ± 0.48	4.03 ± 0.95[b]	132.95 ± 14.58	6.02 ± 1.52[b]	191.43 ± 29.46
	αMPT (300 μg/g; n = 5)	6.37 ± 2.13[f]	11.77 ± 3.53[b]	192.64 ± 11.24[c]	12.64 ± 5.46[b]	220.57 ± 68.97

[a] Mean ± SE.
[b] Significantly different from pretreatment (0 hr) value, $P < 0.05$ (two-sided signed-ranks or paired t test).
[c] $P < 0.05$ (two-tailed U test) compared to control.
[d] $P < 0.02$ (two-tailed U test) compared to control.
[e] $P < 0.01$ (two-tailed U test) compared to control.
[f] $P < 0.05$ (one-tailed U test) compared to control.

TABLE 5
EFFECTS OF INTRAPERITONEAL INJECTION OF CARBIDOPA (50 μg/g) ON SERUM GtH CONCENTRATIONS IN FEMALE GOLDFISH (16L:8D, 12°)

Gonadal condition	Treatment	GtH at times postinjection					
		0 hr	6 hr		24 hr		
		ng/ml	ng/ml	% 0 hr	ng/ml	% 0 hr
Early recrudescence Oct. 1980	Control ($n = 7$)	2.64 ± 0.59[a]	1.58 ± 0.49[b]	59.36 ± 6.71	1.01 ± 0.11[b]	42.68 ± 3.71
	Carbidopa ($n = 5$)	1.35 ± 0.15[c]	1.01 ± 0.12[b]	74.49 ± 2.65	0.88 ± 0.13[b]	66.41 ± 9.38[c]
Midrecrudescence Feb. 1980	Control ($n = 8$, 0 and 24 hr; $n = 5$, 6 hr)	5.89 ± 1.07	6.00 ± 0.96	89.34 ± 15.93	5.28 ± 2.15	103.74 ± 4.28
	Carbidopa ($n = 8$)	4.87 ± 0.53	5.81 ± 0.80[b]	120.05 ± 11.71[d]	12.92 ± 5.25[b,e]	266.49 ± 26.85[e]

[a] Means ± SE.
[b] Significantly different from pretreatment (0 hr) value, $P < 0.05$ (two-sided signed-ranks or paired t test).
[c] $P < 0.05$ (two-tailed U test) compared to control.
[d] $P < 0.02$ (two-tailed U test) compared to control.
[e] $P < 0.002$ (two-tailed U test) compared to control.

ing the injections of 6-OHDA (Table 3) suggests that at least some catecholaminergic neurons inhibit GtH release in goldfish. 6-OHDA does not cross the blood–brain barrier in mammals (Gilman *et al.*, 1980), and is presumed not to do so in goldfish also. However, the goldfish pituitary lies outside of the blood–brain barrier, and therefore it is likely that the catecholamine terminals affected by 6-OHDA are located in the pituitary. This suggests that there is some direct catecholaminergic inhibitory influence over the gonadotrophs of goldfish, although this does not preclude other routes of catecholaminergic control. The failure of 6-OHDA to elevate serum GtH in the latter part of the 3-week treatment period (Table 3) might have resulted from a compensatory decrease in stimulatory input on GtH release following the chronic loss of the inhibitory catecholaminergic influence.

Proceeding on the assumption that the synthetic pathways for catecholamines in teleosts and mammals are similar, an attempt was made to identify the catecholamine responsible for inhibiting GtH release in goldfish by using drugs capable of blocking specific enzymes in the mammalian catecholamine synthetic pathway. Blockage of L-DOPA formation by two different doses of αMPT elevated GtH levels in the serum of fish in either early or late ovarian recrudescence (Table 4). Inhibiting the conversion of L-DOPA to DA by carbidopa elevated serum GtH values in sexually regressed females and in females at midovarian recrudescence (Table 5). However, under the same time course, blocking the production of NE from DA with DDC did not alter circulating GtH concentrations (Table 6). As carbidopa is a peripheral dopa-decarboxylase inhibitor (Gilman *et al.*, 1980), these results suggest that DA acts directly on the pituitary to inhibit GtH release in the goldfish. Direct synaptic contacts between aminergic terminals and gonadotrophs in goldfish has

TABLE 6
EFFECTS OF INTRAPERITONEAL INJECTION OF DDC (2.5 μg/g) ON SERUM GtH CONCENTRATION IN FEMALE GOLDFISH (16L:8D, 12°)

Gonadal condition	Treatment	GtH (ng/ml) 0 hr	6 hr	24 hr
Regressing June 1980	Control (n = 8)	5.03 ± 1.38[a]	3.99 ± 0.86	5.08 ± 0.91
	DDC (n = 7)	3.52 ± 0.53	3.82 ± 0.55	3.92 ± 0.81
Midrecrudescence Feb. 1980	Control (n = 6)	3.65 ± 1.54	13.93 ± 6.45[b]	8.28 ± 2.79[b]
	DDC (n = 5, 0 and 6 hr; n = 4, 24 hr)	3.06 ± 0.51	8.79 ± 1.28[b]	4.68 ± 0.52[b]

[a] Mean ± SE.
[b] Significantly different from pretreatment (0 hr) value, $P < 0.05$ (two-sided signed-ranks or paired t test).

been observed (Kaul and Vollrath, 1974; Leatherland, 1972) and the ability of DA to act directly on rainbow trout pituitaries to decrease GtH secretion has been demonstrated (Crim, 1982). DA can also inhibit LH secretion in humans and rabbits by altering the effects of LHRH at the pituitary level (Judd *et al.*, 1978; Dailey *et al.*, 1978). In contrast, DA lowers LH release in rats by inhibiting pulsatile LHRH secretion (Gallo, 1980, 1981). Recently, Peter and Paulencu (1980) proposed that the preovulatory surge of GtH secretion in goldfish may be regulated, at least in part, by release from the inhibition of GRIF. The presence of a direct dopaminergic inhibitory influence over the gonadotrophs in female goldfish at various stages of gonadal recrudescence and oocyte maturation, as indicated by this study, suggests that DA has GRIF activity.

Results with the α-agonist, clonidine (Gilman *et al.*, 1980) indicate that stimulation of an α-adrenergic receptor may increase GtH release in goldfish (Table 7). The α-adrenergic transmitter, NE, is a likely candidate for mediating this α-adrenergic stimulated GtH release. NE in-

TABLE 7
EFFECTS OF INTRAPERITONEAL INJECTION OF CLONIDINE (30 μg/g) ON SERUM GtH CONCENTRATIONS IN FEMALE GOLDFISH (16L:8D, 12°)

Gonadal condition	Treatment	GtH (ng/ml) 0 hr	6 hr	24 hr
Early recrudescence Oct. 1980	Control (n = 7, 0 and 24 hr; n = 6, 6 hr)	3.05 ± 1.35[a]	1.47 ± 0.24[b]	1.25 ± 0.19[b]
	Clonidine (n = 7, 0 and 6 hr; n = 6, 24 hr)	1.80 ± 0.36	2.34 ± 0.68	2.59 ± 0.78[c]
Midrecrudescence Feb. 1980	Control (n = 8, 0 and 24 hr; n = 5, 6 hr)	5.89 ± 1.07	6.00 ± 0.96	5.28 ± 0.76
	Clonidine (n = 3, 0 hr; n = 6, 6 and 24 hr)	3.35 ± 0.43	4.01 ± 0.52	11.28 ± 1.25[d]

[a] Means ± SE.
[b] Significantly different from pretreatment (0 hr) value, $P < 0.05$ (two-sided signed-ranks test).
[c] Greater than control ($P < 0.05$, one-tailed U test).
[d] Different from control ($P < 0.01$, two-tailed U test).

creases the secretion of LHRH in mammals via α-receptors (Ojeda and McCann, 1973; Sawyer, 1979) and stimulates cAMP production in the pars distalis of goldfish *in vitro* (Deery, 1975). Although the observation by Deery (1975) argues for a direct action on the pituitary, other hypothalamic centers have to be considered as possible sites of action for NE as clonidine penetrates the blood–brain barrier in mammals (Gilman *et al.*, 1980) and is assumed to do so in teleosts.

DDC depressed plasma LH levels in mammals (Kalra and McCann, 1973) but had no effect on circulating GtH concentrations in the present study (Table 5). This conflicts with the suggestion that NE stimulates GtH release in goldfish. The ineffectiveness of DDC injections reported here might be due to the lower dose of DDC used (Table 1) than that normally reported in mammalian experiments (Kalra and McCann, 1973); experiments using higher doses of DDC were tried, but such doses of DDC proved to be fatal to the goldfish (unpublished observations). The possible role of NE requires further confirmation, but the seemingly conflicting results with DDC injection can be also explained if NE acts centrally and presynaptically, via α-receptors, to increase the release of GnRH in goldfish as in mammals (Negro-Vilar *et al.*, 1979; Tima and Flerko, 1974). Stimulation of these α-receptors by clonidine would result in an increase of GnRH release, leading to an elevation of serum GtH concentration as observed (Table 7). Blockage of NE synthesis by DDC would remove the α-stimulation but might not decrease the basal GnRH secretion, causing the serum GtH level to remain similar to that in control animals (Table 6).

In the present study, serum GtH concentrations measured at various times after vehicle injection in fish in a regressed ovarian condition or in early stages of ovarian recrudescence are consistently lower than pretreatment values, while in females in more advanced stages of ovarian recrudescence serum GtH levels of the controls are often elevated (Tables 2, 6–7). Since we have found similar changes in other experiments using different vehicles, and no differences in the GtH concentrations between uninjected and vehicle-injected controls in experiments using fish at both early and late ovarian recrudescence (unpublished results), the changes in serum GtH levels in the vehicle-injected controls in the present study cannot be attributed to the effects of the vehicle. This suggests that stress suppresses GtH release in goldfish with regressed ovaries or ovaries in early recrudescence, but not in female goldfish at later stages of ovarian recrudescence. Seasonal variations in blood levels of corticosteroid hormone in teleosts have been described (Peter *et al.*, 1978). Other experiments, beyond the scope of this study, are required to elucidate the cause of this seasonal difference in response to stress.

An assumption central to the interpretation of the results is that the drugs used function in a similar way in the goldfish as in mammals. Except for reserpine and 6-OHDA (Zambrano, 1975), the effectiveness and actions of these drugs on catecholaminergic neurons in fish have not been investigated. Although caution is justified in interpreting the results, the conclusions drawn from this study on the possible actions of DA and NE on GtH release have been confirmed (unpublished results; for preliminary report see Peter *et al.*, 1982). Experiments are also in progress to further investigate and confirm the role of DA and NE in the neuroendocrinal regulation of GtH release in goldfish.

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Effects of Dopamine on Gonadotropin Release in Female Goldfish, *Carassius auratus*

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Key Words. Dopamine · Gonadotropin · Goldfish · Apomorphine · Pimozide · Preoptic lesion · GnRH

Abstract. Intraperitoneal injections of dopamine (DA) or its agonist, apomorphine decreased and pimozide, a DA antagonist, increased serum gonadotropin (GtH) levels in normal female goldfish. Injection of DA into the third cranial ventricle did not alter serum GtH concentrations. Intraperitoneal injections of DA or apomorphine each reduced the highly elevated serum GtH levels caused by preoptic lesions which abolish an inhibitory hypothalamic influence on GtH release allowing prolonged spontaneous release of GtH. Intraperitoneal injections of DA or apomorphine blocked the stimulation of GtH release induced by injections of a luteinizing hormone-releasing hormone analogue. These results indicate that DA has GtH release-inhibitory activity by actions directly on gonadotrophs to inhibit spontaneous secretion of GtH, and by blocking the actions of the GtH-releasing hormone.

Studies on various mammalian species have shown that certain neurotransmitters may influence the secretion of luteinizing hormone (LH) [2, 20, 22, 32, 36]. It is generally believed that in mammals dopamine (DA) influences LH release by altering the secretion of luteinizing hormone-releasing hormone (LHRH) [12, 13]. However, in rabbits [9] and humans [17], DA has also been shown to directly inhibit the actions of LHRH.

In certain teleost species the gonadotrophs are directly innervated by both peptidergic and aminergic neurons [1, 18, 21, 28], providing the possibility that their activity can be controlled directly by the actions of two hypothalamic factors. The presence in the hypothalamus of teleosts of a gonadotropin (GtH)-releasing hormone, that is similar to LHRH, and an unidentified GtH release-inhibitory factor have been demonstrated [27, 28, 31]. In goldfish, in vivo experiments with drugs capable of inhibiting specific enzymes of catecholamine synthesis and neural activities suggest that DA may inhibit, while an alpha-adrenergic mechanism may stimulate, GtH release [6]. However, whether the level of action of these neurotransmitters was directly on the gonadotrophs or the brain, to influence the release of neurohormones, was not clear from this work.

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In the present study, the possible role of DA in the neuroendocrine regulation of GtH release in female goldfish was investigated by determining the effects of intraperitoneal injections of DA, its agonist, apomorphine, and the DA antagonist, pimozide, on serum GtH concentrations in normal goldfish. The possible involvement of hypothalamic factors in mediating the actions of DA on GtH release was also tested by studying the effects on serum GtH concentrations of intracranial injections of DA in normal fish, and intraperitoneal injections of DA and apomorphine in fish injected with a LHRH analogue. The actions of DA in fish in which the influence of GtH release-inhibitory factor was abolished by lesions in the preoptic region [31] were also investigated.

Materials and Methods

General

Goldfish (comet or common variety; body weight, b.w. = 27.3 ± 6.9 g, mean ± SD), purchased from Grassyforks Fisheries Co., Martinsville, Ind., were held on a 12 h light:12 h dark (12L:12D; light on at 08.00) photoperiod in flow-through aquaria (150- or 225-liter) at 12 °C for a minimum of 7 days after arrival. 7 days before an experiment, the photoperiod was usually extended to 16L:8D; lengthening the photoperiod serves to accelerate gonadal

recrudescence in goldfish [26]. Fish were fed daily, ad libitum, with Ewos trout pellets.

Dopamine HCl, apomorphine HCl, and des Gly ^{10}[D-Ala^6] LHRH ethylamide (LHRH-A) were purchased from Sigma, St. Louis, Mo., USA. Pimozide was a gift from Janssen Pharmaceuticals Ltd., Beerse, Belgium. DA, apomorphine, and pimozide were made up fresh before injection in a vehicle of acidified 0.7% NaCl with 0.1% sodium metabisulfite. Apomorphine and DA were injected as a solution while pimozide was injected as a suspension. LHRH-A was dissolved in fish physiological saline (PS) [5]. Control groups were given an equivalent volume of vehicle and/or PS.

Fish were anesthetized by immersion in 0.05% tricaine methanesulfonate (Sigma) before handling. Prior to each experiment, the fish were weighed and identified with a numbered metal tag attached to the operculum. Blood of each fish was sampled serially by puncturing the caudal vasculature with a 25-gauge needle attached to a 1.0-ml disposable syringe. Blood samples were allowed to clot on ice for several hours, the serum separated by centrifugation, and stored at –28 °C. Serum GtH levels were determined by radioimmunoassay [8, 14, 15]. After each experiment, fish were killed and the gonadosomatic index (GSI) and the stage of ovarian maturation determined [6].

Signed-ranks test or paired t test were used to compare pretreatment and posttreatment serum GtH levels within experimental groups. Mann-Whitney U test was used to compare GtH concentrations between the experimental and control groups [37].

Effects of Intraperitoneal Injections of DA, Apomorphine, and Pimozide on Serum GtH Levels in Normal Goldfish

In separate experiments, DA (10 and 100 μg/g b.w.), apomorphine (20 μg/g b.w.) and pimozide (10 μg/g b.w.) were injected into female goldfish undergoing ovarian recrudescence (GSI = 3.30 ± 1.84%, mean ± SD). Blood samples were taken just prior to drug injections and at 1 h after DA injection, or 6 and 24 h after apomorphine or pimozide injections.

Effects of Intraperitoneal Injections of DA and Apomorphine on Serum GtH Levels in Preoptic-Lesioned Fish

Female goldfish undergoing ovarian recrudescence (GSI = 6.99 ± 3.01%) were used. Electrodes for lesioning and sham operation were No. 0 stainless steel insect pins insulated to within 0.5–0.6 mm of the tip with Insul-X. Large lesions were made in the anterior-ventral preoptic region (electrode coordinates: +1.3, M, D 2.3–2.4) with radiofrequency current at 90 V for 30 s according to procedures outlined by *Peter and Gill* [29]. Electrodes were lowered but no current was applied in sham-operated fish. DA (10 and 100 μg/g b.w.) was injected into preoptic-lesioned fish on day 2 postlesioning, blood samples were taken just before and at 2 h after DA injection. Apomorphine (20 μg/g b.w.) was injected into preoptic-lesioned fish at day 1 postlesion; blood samples were taken just before and at 24 h after apomorphine injection. The brains of preoptic-lesioned or sham-operated fish were fixed in Bouin's solution, embedded in paraffin, serially sectioned at 7 μm, stained with paraldehyde fuchsin, and counterstained with acid fuchsin, poucean xylidine and fast green [29]. The placement of the lesions or the electrodes were then checked under a light microscope. The photoperiod was not extended to 16L:8D for the DA-injection experiment.

Effects of Intraventricular Third Cranial Ventricle Injections on Serum GtH Concentrations in Normal Fish

Female goldfish undergoing ovarian recrudescence (GSI = 5.04 ± 3.08%) were used. DA (2 and 20 μg; injection volume = 2 μl) was injected into the third cranial ventricle at the level of the preoptic area (coordinates: +0.8, M, D 2.0) [29]. Blood samples were taken just prior to and at 2 h after DA injection. The brains of these fishes were fixed in Bouin's solution and the placement of the injection needle checked by histology as described in the previous section.

Effects of Intraperitoneal Injections of DA, and Apomorphine on Serum GtH Concentrations in LHRH-A Injected Goldfish

Female goldfish (GSI = 9.00 ± 3.86%) were used. LHRH-A (0.1 μg/g b.w.) was injected intraperitoneally twice with a 12-hour interval (first injection at 21:00). DA (10 and 100 μg/g b.w.) was injected into LHRH-A injected fish at 4 h after the second LHRH-A injection; blood samples were taken just before and at 2 h after the DA injection. In another experiment, apomorphine (10 and 20 μg/g b.w.) was injected simultaneously with both of the LHRH-A injections. These fish were bled at 6 and 24 h after the second LHRH-A injection.

Results

Effects of Intraperitoneal Injections of DA, Apomorphine, and Pimozide on Serum GtH Levels in Normal Fish

As shown in table I, injection of DA caused a significant decrease in serum GtH concentrations at 1 h postinjection compared to pretreatment and control values, when expressed either as absolute values or as percent of the pretreatment values. The serum GtH levels at 1 h after DA or vehicle injection were expressed as percent of the pretreatment values because there was a significant difference in the pretreatment GtH values between the vehicle-injected control and the high-dose DA group (table I). Injection of apomorphine prevented the increase in serum GtH levels observed in the controls at 6 and 24 h postinjection, and significantly decreased serum GtH concentrations at 24 h postinjection compared to the control values (table I). Injection of pimozide prevented the decrease in serum GtH levels observed in the controls at 6 and 24 h postinjection, and also increased the serum GtH values significantly above those of the controls and pretreatment values by 24 h postinjection (table I).

Effects of Intraperitoneal Injections of DA and Apomorphine on Serum GtH Levels in Preoptic-Lesioned Fish

Lesions in the preoptic area destroyed the nucleus preopticus periventricularis, the nucleus preopticus, and, in some cases, the anterior-dorsal region of the nucleus anterioris periventricularis (see *Peter and Gill* [29] for nomenclature). Fish bearing such lesions had significantly higher serum GtH concentrations than sham-operated fish at both days 1 and 2 postlesioning (table II), similar to previous re-

Table I. Effects of intraperitoneal injections of DA, apomorphine (APO), and pimozide (PIM) on serum GtH levels in normal female goldfish

Treatments	n	GtH at times postinjection[1]				
		0 h	1 h		6 h	24 h
		ng/ml	ng/ml	%0 h	ng/ml	ng/ml
(A) vehicle	5	3.0 ± 0.3	5.0 ± 0.5[3]	165 ± 15.6[3]		
DA, 10 μg/g b.w.	6	3.5 ± 0.1	2.6 ± 0.2[4, 5]	73.3 ± 5.4[4, 5]		
DA, 100 μg/g b.w.	4	4.2 ± 0.4[2]	3.4 ± 0.3[4, 5]	83.2 ± 3.9[4, 5]		
(B) vehicle	6	3.2 ± 0.4			4.0 ± 0.5[3]	4.8 ± 0.9[3]
APO, 20 μg/g b.w.	4	3.1 ± 0.2			3.5 ± 0.7	3.4 ± 0.2[6]
(C) vehicle	6	5.0 ± 0.7			3.5 ± 0.4[4]	3.2 ± 0.2[4]
PIM, 10 μg/g b.w.	6	4.4 ± 0.7			3.4 ± 0.3	13.3 ± 7.5[7, 8]

[1] Mean ± SE.
[2] Significantly greater than vehicle-injected group, $p < 0.025$.
[3] Significantly greater than pretreatment value, $p < 0.025$.
[4] Significantly less than pretreatment value, $p < 0.025$.
[5] Significantly less than vehicle-injected controls, $p < 0.01$.
[6] Significantly less than vehicle-injected controls, $p < 0.025$.
[7] Significantly greater than pretreatment, $p < 0.05$.
[8] Significantly greater than vehicle-injected controls, $p < 0.01$.

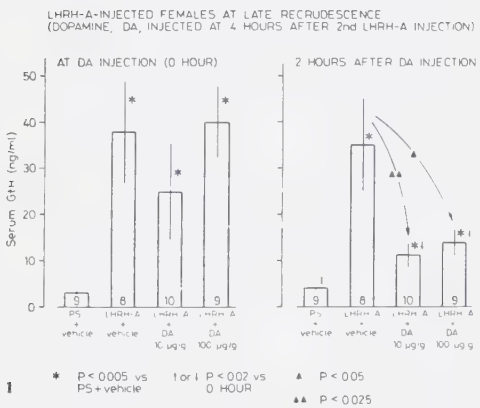
Table II. Effects of intraperitoneal injections of DA and apomorphine (APO) on serum GtH levels of preoptic-lesioned female goldfish held at 12 °C

Treatments	n	GtH at times postinjection[1]				
		0 h	2 h		24 h	
		ng/ml	ng/ml	%0 h	ng/ml	%0 h
(A) vehicle[2]	5	183.1 ± 44.8[4]	146.4 ± 36.6	89.7 ± 19.9		
DA, 10 μg/g b.w.	6	397.5 ± 117.2	150.9 ± 22.4[5]	43.1 ± 6.7[6]		
DA, 100 μg/g b.w.	6	165.9 ± 56.8[4]	109.8 ± 36.0[5]	56.6 ± 8.3[6]		
(B) vehicle[3]	7	497.7 ± 134.5			371.6 ± 104.9	72.6 ± 17.9
APO, 20 μg/g b.w.	12	380.7 ± 58.9			22.4 ± 2.3[7]	8.9 ± 2.7[7]

[1] Mean ± SE.
[2] At the time of vehicle or DA injection, the preoptic-lesioned fish had serum GtH concentrations (252.7 ± 52.3 ng/ml; n = 17) that were significantly higher ($p < 0.001$) than those (10.9 ± 1.5 ng/ml; n = 8) of sham-lesioned fish in a similar stage of ovarian maturation.
[3] At the time of vehicle or apomorphine injection, the preoptic-lesioned fish had serum GtH concentrations (423.8 ± 61.2 ng/ml; n = 19) that were significantly higher ($p < 0.001$) than those (11.0 ± 1.5 ng/ml; n = 5) of sham-lesioned fish in a similar stage of ovarian maturation.
[4] Significantly less than values in the group injected with DA at 10 μg/g b.w. $p < 0.05$.
[5] Significantly less than pretreatment, $p < 0.025$.
[6] Significantly less than pretreatment and vehicle-injected controls, $p < 0.025$.
[7] Significantly less than pretreatment and vehicle-injected controls, $p < 0.001$.

Table III. Effects of intraventricular (third cranial ventricle) injections of DA on serum GtH concentrations in normal female goldfish held at 12 °C (mean ± SE)

Treatments	n	GtH (ng/ml) at times postinjection	
		0 h	2 h
Vehicle	7	5.2 ± 0.9	4.4 ± 0.4
DA, 2 µg	8	6.1 ± 1.7	5.7 ± 0.9
DA, 20 µg	9	5.0 ± 0.8	5.3 ± 1.1



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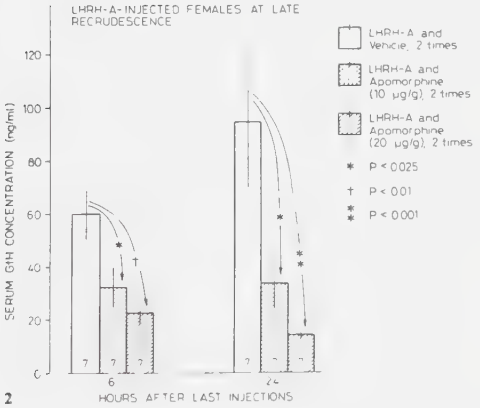

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results [27, 31]; characteristic of the effect of such lesions, there was a large range in the serum GtH levels found at 1 and 2 days postlesion (see below). DA injections at either 10 or 100 µg/g b.w. significantly decreased the elevated serum GtH concentrations in preoptic-lesioned fish at 2 h postinjection, compared to pretreatment values (table II). Apomorphine injection significantly lowered the serum GtH values in preoptic-lesioned fish at 24 h postinjection, compared to both control and pretreatment values (table II). Since the serum GtH concentrations in preoptic-lesioned goldfish were variable (table II), the postinjection serum GtH concentrations were also expressed as percent of the preinjection values. When the results were expressed as percent of the preinjection values, injections of DA or apomorphine significantly lowered the serum GtH values in preoptic-lesioned fish compared to both control and preinjection values (table II).

Effects of Intraventricular (Third Cranial Ventricle Injections of DA on Serum GtH Concentrations in Normal Fish

Intraventricular injection did not damage cells of the preoptic area surrounding the injection site. There were no effects of intraventricular injections of DA on serum GtH concentrations at 2 h postinjection (table III).

Effects of Intraperitoneal Injections of DA, and Apomorphine on Serum GtH Concentrations in LHRH-A Injected Goldfish

Female goldfish given two injections of LHRH-A, with a 12-hour interval, had significantly higher serum GtH concentrations than vehicle-injected controls at 4 and 6 h after the second LHRH-A injection (fig. 1). Serum GtH concentrations in fish injected with LHRH-A followed by DA were significantly lower than those in fish receiving LHRH-A and the vehicle for DA (fig. 1). In the LHRH-A treated fish DA injection also significantly decreased serum GtH concentrations at 2 h compared to preinjection levels in the same fish. However, DA injections did not lower serum GtH levels in fish previously treated with LHRH-A to levels found in the PS and vehicle-injected fish. Goldfish injected with apomorphine at both LHRH-A injections had serum GtH values that were significantly lower than those injected with LHRH-A and the apomorphine vehicle at 6 and 24 h after the second LHRH-A injection (fig. 2).

Fig. 1. Effects of intraperitoneal injections of DA on serum GtH concentrations in female goldfish held at 12 °C and given two injections of LHRH-A (means ± SE are plotted).

Fig. 2. Effects of intraperitoneal injections of apomorphine, administered simultaneously with both the first and the second LHRH-A injections, on serum GtH concentrations in female goldfish held at 12 °C (mean ± SE are plotted).

Discussion

Results of a previous study, using drugs which alter various steps in the synthetic pathway of catecholamines, indicated that DA inhibits GtH secretion in the goldfish [6]. In the present study, intraperitoneal injections of DA and its agonist, apomorphine, decreased while the DA antagonist, pimozide, increased serum GtH levels, consistent with previous findings. Although the effects of DA on LH release in mammals are still controversial [2, 20, 22, 32, 36], systemic applications of DA and its agonists have been shown to decrease while DA antagonists increase LH secretion in rats and humans [10, 11, 33–35, 38]. Although it is believed that DA alters LH secretion in mammals by acting centrally to modify LHRH release [12, 13], a pituitary site of action has also been suggested in rabbits [9] and humans [16, 17].

Although some dopaminergic receptor blockers also have the ability to block alpha-adrenergic receptors in mammals, the stimulatory influence of pimozide on GtH release in goldfish does not seem to be mediated by alpha-receptor blockage. Blockage of alpha-receptors would decrease serum GtH concentrations in goldfish since stimulation of alpha-receptors elevated serum GtH levels [6]. Apomorphine and pimozide had a more prolonged action on serum GtH levels than DA (table I). DA agonists and antagonists have a higher affinity to DA receptors than DA [19] and may be metabolized at a slower rate than DA. The apparently slower onset of the GtH response to apomorphine or pimozide injections relative to that observed in mammals may be the result of the lower body temperature of the goldfish (12 °C) used in the present study. When the water temperature was increased from 12 to 20 °C, the GtH response to intraperitoneal injections of pimozide could be observed as early as 3 h postinjection [*Sokolowska and Peter*, unpubl. results]. Similarly, the GtH response to intraperitoneal injections of apomorphine could also be advanced by increasing the water temperature to 21 °C [*Chang and Peter*, unpubl. results].

A blood-brain barrier exists for DA in mammals [3]. However, the presence of such a barrier has not been demonstrated in teleosts. Therefore, it is possible that DA may act directly on the pituitary and/or the hypothalamus to alter GtH release in the goldfish. However, third-ventricle injections of DA did not alter serum GtH concentrations in goldfish at 2 h postinjection (present study), although serum growth hormone levels were increased in these same fish [*Cook and Chang*, unpubl. results]. In preoptic-lesioned goldfish, intraperitoneal injection of DA was effective in reducing serum GtH levels at 2 h postinjection. When viewed together these results suggest that the actions of DA on GtH release in goldfish are not likely due to central action.

The influence of the GtH release-inhibitory factor goldfish can be abolished by lesions in the preoptic region or destruction of the pituitary stalk, resulting in a prolonged massive spontaneous release of GtH [31]. As in previous studies [31], preoptic lesions significantly elevated serum GtH concentrations. Intraperitoneal injections of DA and apomorphine each significantly reduced the elevated serum GtH concentrations in preoptic-lesioned female goldfish. These results indicate that DA can act directly on the pituitary as a GtH release-inhibitor to block the spontaneous release of GtH.

The possible interaction of GtH-releasing hormone and DA in the regulation of GtH release in goldfish was tested by studing the effects of DA and apomorphine on serum GtH concentrations in goldfish injected with a superactive analogue of LHRH. The analogue was effective in stimulating GtH release in female goldfish when administered as two intraperitoneal injections, 12 h apart, as in previous experiments using male goldfish [25]; with this schedule the first injection is thought to potentiate the action of the second injection. DA injected intraperitoneally after the second LHRH-A injection decreased, at 2 h after DA administration, the elevated serum GtH levels induced by LHRH-A. Apomorphine injected simultaneously with both LHRH-A injections blocked the LHRH-A-induced increase in serum GtH levels. These results indicate that DA modulates the response to GtH-releasing hormone in goldfish, in some cases effectively shutting off the GtH release-response. The ability of DA to block the actions of LHRH has also been demonstrated in rabbits [9] and humans [16, 17]. LHRH is believed to stimulate GtH release via activation of adenylate cyclase [24]. DA receptors have been found to be negatively coupled to adenylate cyclase in rat pituitary cells [23]; perhaps DA blocks the actions of the GtH-releasing hormone by interfering with the formation of adenosine cyclic monophosphate.

Direct dynaptic contracts between aminergic terminals and gonadotrophs in goldfish have been observed [18, 21]. Although *Breton* et al. [4] reported that DA did not alter GtH release from carp pituitaries in vitro, preliminary results have shown that DA decreased the spontaneous and LHRH-A-stimulated GtH release from dispersed goldfish pituitary cells in vitro [*MacKenzie and Chang*, unpubl. results]. *Crim* (unpubl. results cited in an abstract [7]) also reported that DA inhibited the basal and LHRH-stimulated release of GtH from rainbow trout pituitaries in vitro.

In the present study, the effects of DA, apomorphine or pimozide injections on serum GtH levels were demonstrated in female goldfish with GSI values ranging from less than 1% to over 20%. This suggests that the ability of DA to inhibit GtH release is not restricted to only a short period in the reproductive cycle of the female goldfish. However, the magnitude of the GtH response to intraperitoneal injections of DA or apomorphine was smaller in intact than in preoptic-lesioned goldfish. These results suggest that in normal intact goldfish, the inhibition on GtH secretion is near maximum, and the ability of the gonadotrophs to further de-

crease GtH release in response to an injection of DA or apomorphine is limited.

In the present work, the serum GtH levels in the vehicle-injected controls were in some cases elevated, and in one experiment depressed, relative to preinjection values. These differences cannot be readily explained, although similar occurrences have been observed in previous work [6]. However, perhaps the GtH release-response to stress may change as the fish progresses through ovarian recrudescence. In female goldfish in very early stages of ovarian recrudescence (GSI = 2.26 ± 0.30%, mean ± SE, as for the fish in table I, C) the serum GtH levels of the vehicle-injected controls decreased, whereas in fish at somewhat later stages of ovarian recrudescence (GSI = 2.82 ± 0.32% and 4.89 ± 0.54%, mean ± SE, as for the fish in table I, A and B, respectively) serum GtH levels increased. Seasonal variations in blood levels of corticosteroid hormones in teleosts have been described [30]. Since we have not found any difference in serum GtH concentrations between noninjected and vehicle-injected controls (data not shown), and have observed similar changes in other experiments using different vehicles (unpubl. data), the changes in serum GtH levels in the vehicle-injected controls in the present study cannot be attributed to the effects of the vehicle. Other experiments, beyond the scope of the current study, are required to clarify the seasonal changes in GtH release in goldfish in response to stress.

In summary, the present experiments indicate that DA has GtH release-inhibitory activity in goldfish by actions directly on gonadotrophs to inhibit the spontaneous release of GtH, and by blocking the actions of the GtH-releasing hormone. Experiments are now in progress to further investigate the role of DA in the neuroendocrine regulation of the preovulatory surge of GtH in goldfish, a process which may be regulated by stimulation of GtH release by releasing hormone and a release from the GtH release-inhibitory effects of DA. Whether other factors, in addition to dopamine, may have GtH release-inhibitory activity in goldfish remains to be determined.

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Appendix III. A Preliminary Study on The Effects of Intraperitoneal Injection of Clonidine and L-DOPA on Serum GtH Concentrations in Goldfish.

A. Materials and Methods

The procedures described in Chapter II for photoperiod-temperature regime and procedures for fish maintenance, fish handling, blood samplimg and radioimmunoassay were followed. L-DOPA HCl (L-DOPA, Sigma) and clonidine, dissolved in a vehicle of acidified 0.7% saline with 0.1% sodium metabisulfite, were injected intraperitoneally (dosages given in Table A3.1) in goldfish (mixed sex). Control fish were injected with an equivalent volume of vehicle. Blood samples were taken at various times after drug or vehicle injection (see Table A3.1 for details). Serum GtH concentrations between experimental and control groups were compared using Mann-Whitney U test as described in Chapter II. The GSI of experimental fish was not determined in these experiments.

B. Results

Serum GtH concentrations were significantly higher in fish injected with clonidine (300 ug/g) than in controls at 2 and 6 h postinjection (Table A3.1, A). Fish injected with L-DOPA (100 ug/g) had significantly lower serum GtH levels than controls at 1 and 2 h post-injection (Table A3.1, B, C).

Table A3.1. Effects of intraperitoneal injection of clonidine or L-DOPA on serum GtH concentrations in goldfish.

Experimental Condition	Treatment	n	Serum GtH (ng/ml) at h postinjection		
			1	2	6
A)[1]					
12L:12D; 12°C	vehicle	8		5.3±1.5[2]	3.1±0.8
Jan.	clonidine, 300 ug/g[3]	8		19.3±7.0[4]	26.7±9.7[5]
B)[1]					
12L:12D; 12°C	vehicle	8		5.8±1.0	3.5±0.5
Feb.	L-DOPA, 100 ug/g	8		3.1±0.7[6]	2.9±0.9
C)					
16L:8D; 12°C	vehicle	12	5.5±2.2		
April	L-DOPA, 100 ug/g	10	3.6±1.9[6]		

1 Photoperiod not extended to 16L:8D prior to experiment.

2 Mean±SE.

3 Doses in ug/g body weight.

4 Significantly greater than vehicle injected controls, $P<0.025$.

5 Significantly greater than vehicle injected controls, $P<0.005$.

6 Significantly less than vehicle injected control, $P<0.025$.

Appendix IV. Effects of Intraperitoneal Injection of Apomorphine and Pimozide on Serum Gonadotropin Concentrations in Female White Suckers, *Catostomus commersoni*.

A. Materials and Methods

Adult female white suckers were captured at a small stream draining into Lac Ste. Anne, Alberta during their spawning migration in May 1982. An initial blood sample was taken from the caudal vasculature of each fish with a 21-gauge needle attached to a disposable 3-ml syringe within 10 minutes of netting. Fish were identified with individual fin clips, injected intraperitoneally with pimozide (10 mg/fish), apomorphine (20 mg/fish) or the drug vehicle (acidified 0.7% saline with 0.1% sodium metabisulfite) as descibed in Chapter III, and transferred to one of two open-mesh cages (180 cm X 120 cm X 60 cm, L X W X H; 12 fish per cage) anchored in the stream. All fish were blood sampled again at 1, 3 and 8 days after injection. Blood samples were allowed to clot on ice for several hours and serum separated by centrifugation. Serum samples were kept on dry ice and transported back to the laboratory for storage at -28°C. Serum GtH levels were assayed by radioimmunoassay as described in Chapter IV. At the end of the experiment, fish were killed by a sharp blow to the base of the skull. Serum GtH levels between experimental and vehicle injected control groups were compared using the Mann-Whitney U test as described in Chapter II.

B. Results

At 3 days postinjection, serum GtH concentrations in fish injected with pimozide were significantly higher than those of the controls (Table A4.1). Fish injected with apomorphine had significantly lower serum GtH concentrations than vehicle injected controls at 1, 3 and 8 days postinjection (Table A4.1).

Table A4.1. Effects of intraperitoneal injection of pimozide (10 mg/fish) and apomorphine (10 mg/fish) on serum GtH concentrations in female white suckers.[1]

Treatment		Serum GtH (ng/ml) at days postinjection			
	n	0[2]	1	3	8
vehicle	8	2.1±0.4[3]	1.9±0.2	2.8±0.4	3.5±1.3
pimozide	8	1.6±0.2	2.2±0.2	5.8±1.2[4]	4.0±0.8
apomorphine	8	1.7±0.1	1.5±0.1[5]	1.7±0.1[6]	1.6±0.1[6]

[1] Water temperature measured at the time of blood sampling on days 0, 1, 3 and 8 were 10.0, 4.5, 5.0 and 12.5°C, respectively.

[2] Samples taken just prior to injection.

[3] Mean±SE.

[4] Significantly greater than vehicle injected controls, $P<0.025$.

[5] Significantly less than vehicle injected controls, $P<0.05$.

[6] Significantly less than vehicle injected controls, $P<0.025$.